

A detailed 3D illustration of cancer metastasis. It shows a network of orange, branching structures representing blood vessels or lymphatics. Several blue, irregularly shaped masses, representing tumor cells, are shown migrating along these structures. Some blue masses are clustered together, while others are isolated, illustrating the process of cancer spreading from a primary site to secondary locations.

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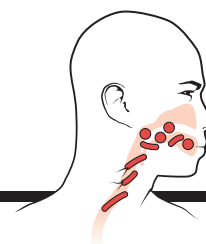
SPECIAL ISSUE

FATAL MIGRATIONS

Frontiers of metastasis
research

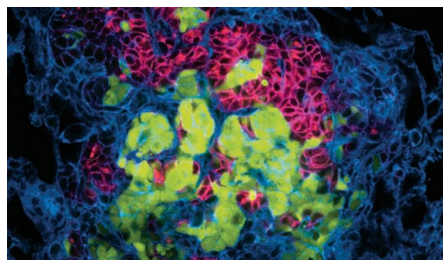
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ON THE COVER



Artist's interpretation of tumor cells in transit from the primary tumor (background) to the site of metastasis (foreground). After entering the vasculature (depicted

as red snaking branches), circulating tumor cells—shown here in a much greater number than would occur in a cancer patient—eventually exit at the metastatic site. Recent research on metastasis, a leading cause of cancer mortality, is highlighted in a special section beginning on page 162.

Illustration: Valerie Altounian/Science

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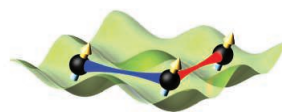
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The transformation of oncology

When I was trained at a prominent New York medical center in the 1960s, cancer was relegated to a minor place in the curriculum. Cancer patients were housed in a separate hospital, rarely visited by students or the medical house staff. If not amenable to surgery or radiotherapy, most cancers were regarded as hopeless. Research on cancer was not accorded the attention received by infectious, endocrine, autoimmune, cardiovascular, or neuropsychiatric disorders.

Now things are dramatically different in research, teaching, and care. Much of the new optimism comes from an understanding of cancer's pathophysiology. Research has shown that cancers are intimately entwined with basic life processes—diseases of the genome, with perturbations of signaling pathways and essential cell functions. New diagnostic categories are based on genetic profiles, not just morphology. Effective prevention occurs through viral vaccines and behavioral changes that reduce exposure to mutagens; screening detects some cancers early enough for curative surgery. Therapeutic strategies are increasingly informed by underlying genomic damage or by the immunological responses to cancer-associated antigens. Such knowledge has driven annual reductions of 1 to 2% in age-adjusted cancer mortality rates in the United States for many years.

These encouraging signs have stimulated local, national, and even global efforts to hasten progress against cancer, with clearer strategies than were possible when President Nixon signed the National Cancer Act in 1971 and launched what is still called the “war on cancer.” President Obama's 2015 decision to devote a third of his Precision Medicine Initiative to oncology was predicated on the new promise of using genomics, informatics, and redesigned clinical trials to revise cancer diagnostics, improve prognostic tools, and develop new therapeutics. The Administration's more recent announcement of a cancer “moonshot,” led by Vice President Biden, aspires to move cancer research more quickly—accomplishing 10 years of work in 5

years—by leaping fences between disciplines, connecting health centers, and interweaving the academic and industrial sectors. Implausible goals that tarnished earlier campaigns, such as the elimination or cure of certain cancers by a certain date—the equivalent of a true moonshot—have been conspicuously absent.

Can such attention from national leaders be helpful without additional funds to support research? After all, despite this year's \$2 billion increase, the budget of the U.S. National Institutes of Health remains about 20% below its value a decade ago, and fundamental problems in cancer biology remain unsolved. One prominent example—how cancer cells metastasize from primary to

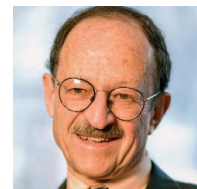
distant sites—is addressed by several articles in this special issue of *Science* (see page 162). Expanded work on such questions requires expanded resources, yet provision of such funds remains uncertain.

Still, there are important things to do, even without enlarged Congressional appropriations. The White House is already using its convening power to encourage greater collaboration, information sharing, and team efforts. The vice president could use his popularity and bully pulpit to move beyond research to emphasize cancer prevention: the life-saving values of vac-

cines for human papilloma and hepatitis B viruses, the avoidance of tobacco use, the control of obesity. The Administration could also exercise its regulatory authority—most potently, to direct the Centers for Medicare and Medicaid Services (CMS) to allow reimbursement for molecular profiling of cancers. That would vastly increase the data available for analysis, accelerate interpretation of genetic profiles, provide a test bed for true sharing of clinical information, and allow future coverage determinations by CMS to be made more quickly and sensibly.

We are living in a remarkable time for cancer research, with synergies among its components and strong backing from the nation's leaders. Let us take advantage of this moment, even without the certainty of additional dollars.

—Harold Varmus



Harold Varmus is the Lewis Thomas University Professor at the Meyer Cancer Center, Weill Cornell Medicine, New York, NY; Senior Associate Member of the New York Genome Center, New York, NY; and former director of the U.S. National Cancer Institute. E-mail: varmus@med.cornell.edu



“Can...attention from national leaders be helpful without additional funds...?”

“What is that a ‘no’ to, the portal or the demons?”

Online query aimed at **Kate Kahle**, who oversees social media at CERN, after she denied that the Large Hadron Collider (LHC) was involved in occult activities. People have speculated that the LHC opens a portal to another dimension or summons the Antichrist, she told *The Wall Street Journal*.



Millions of bats in the eastern United States, such as this one in a Vermont mine, already suffer from white-nose syndrome.

Killer bat fungus hits West Coast

White-nose syndrome, a lethal fungus devastating U.S. bat populations in the East and Midwest, has crossed the Continental Divide for the first time, unexpectedly popping up in Washington—approximately 2000 kilometers farther west than previously seen. First identified in the United States in 2008 in caves near Albany, the fungus *Pseudogymnoascus destructans* is thought to have killed more than 6 million bats in 28 states and five Canadian provinces. It infects bats during hibernation, forming a distinctive halo of white fuzz on the bat's muzzle; it may cause them to burn more energy, become dehydrated, or wake and leave their shelter when it's too cold. Now, the discovery of the fungus in a little brown bat found in mid-March by hikers at the edge of the Cascade Mountains is confirmation of what scientists considered the inevitable spread of the disease across the continent. “This is the news we have been bracing for and warning about going back for the last 8 years,” says Jeremy Coleman, an ecologist and head of the U.S. Fish and Wildlife Service's white-nose syndrome program in Hadley, Massachusetts. <http://scim.ag/batfungusWest>

AROUND THE WORLD

Alarm over fetal tissue probe

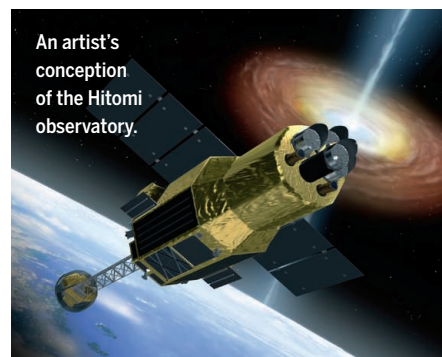
WASHINGTON, D.C. | A special investigative panel in the U.S. House of Representatives is stepping up its probe into the use of fetal tissue in biomedical research with 12 subpoenas aimed at researchers and abortion providers. This second round of inquiries, released last week, deepened concerns that personal information revealed in the investigation could make researchers the targets of extremist violence. The panel, which grew out of Republican backlash after an antiabortion group released undercover videos of Planned Parenthood (*Science*, 18 September 2015, p. 1267), previously asked the University of New Mexico, Albuquerque (UNM), to turn over the names of faculty and students who participated in research using fetal tissue, but

UNM refused. Although recipients' names have been redacted on several of the new subpoenas, UNM confirms that two are addressed to its faculty. A 31 March letter from educational groups, including the Association of American Medical Colleges, urged the panel to set up rules to safeguard personally identifiable information. <http://scim.ag/fetaltissuesubp>

Scientists hunt for Hitomi

TOKYO | Officials of the Japan Aerospace Exploration Agency (JAXA) announced last week that they are still trying to re-establish communications with the wayward Hitomi x-ray observatory, and are analyzing its last transmissions of data for clues about what might have gone wrong. A joint JAXA-NASA mission, Hitomi was launched 17 February; normal

communications were lost on 26 March. Originally called ASTRO-H, Hitomi carries instruments designed to detect x-rays and gamma rays emanating from black holes, swirling gases in galaxy clusters, and supernova remnants. Ground stations intermittently picked up signals apparently



An artist's conception of the Hitomi observatory.

from the spacecraft four times, the last on 29 March. Ground visual and radar observations indicate that the craft has split into at least two pieces and is likely spinning. Speculation about possible causes of an onboard explosion centers on a rupture of the helium tank for an x-ray detector cooling system, fuel leakage from the attitude control engines, and a battery malfunction. <http://scim.ag/Hitomihunt>

Turkish researcher arrested

CAMBRIDGE, MASSACHUSETTS, AND ISTANBUL, TURKEY | A university protest greeted Turkey's science minister during a visit to the United States, just a few days after the country took yet another academic into custody for signing a petition that criticized the government's treatment of the Kurdish minority group. Turkish literary scholar Meral Camcı, one of dozens of academics fired from the country's universities after the petition went out, went to France for vacation several days before Turkey arrested three other signers on 15 March. Camcı vowed to keep protesting against the government (*Science*, 25 March, p. 1381). However, on 31 March, upon returning to Istanbul, she, too, was arrested. In support of her and the others, protesters this week picketed the arrival of Turkey's minister of science, Fikri Işık, at the Massachusetts Institute of Technology in Cambridge; Işık was there as part of an event organized by the Technological Research Council of Turkey to encourage researchers to work in the country.

A new Viking site?

POINT ROSE, CANADA | On a windswept peninsula in southern Newfoundland, a team of archaeologists has discovered what they say is the second known Viking site in North America. The team used satellite images to identify ground features that might be evidence of past human activity—in particular, signs of altered vegetation that might be attributable to changes in soil moisture as a result of human structures. Since November 2015, the team, led by archaeologist Sarah Parcak of the University of Alabama, Birmingham, has conducted a preliminary small-scale dig here. They uncovered clear signs of habitation: a stone iron-working hearth surrounded by what looks like a turf wall. Whether the site was a Viking outpost, however, is less clear, but indigenous peoples in the area were not known to build turf structures, nor were they known to mine bog iron. The only other confirmed Viking site on the continent



Whalers capture a sperm whale in this early 19th century painting.

Sperm whale foreheads designed for ramming

Ever since a sperm whale (*Physeter macrocephalus*) head-butted and sank a whaler's ship in 1821, whalers and scientists have theorized that the mammals' boxy foreheads might be adapted for use as battering rams—possibly for male-on-male battles over access to females. But skeptics point out that structures key to the whale's clicking communication would be front and center in such an impact. In a new study, researchers tested the idea by running virtual crash tests: They simulated ramming impacts on a sperm whale's skull, and recorded where the impacts produce the most mechanical stress. The simulations showed that vertical tissues that divide up the large, oil-filled organ—called the “junk” by whalers—help spread the force of impact over the skull; removing those compartments increased overall stress on the skull by 45%, the authors report this week in the journal *PeerJ*. Male ramming behavior has only been observed once in sperm whales—but, the authors say, these contests may be occurring below the surface.

is on Newfoundland's northern tip, at a site called L'Anse aux Meadows; that settlement, dating to about 1000 C.E., was abandoned after only a few years.

NEWSMAKERS

Scientist accused of perjury

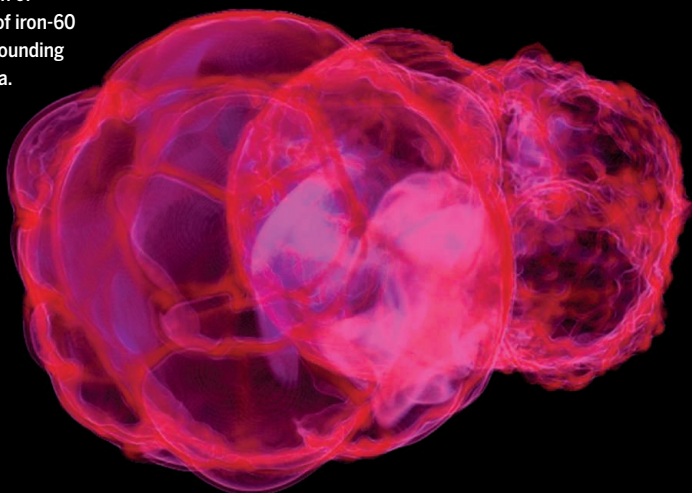
French asthma specialist **Michel Aubier** is in the crosshairs of the French Senate, because he apparently didn't disclose his paid work for an oil company during an inquiry last year into the costs of air pollution. Aubier, a pneumologist at the Hôpital Bichat-Claude Bernard in Paris, told a Senate committee of inquiry that the link between air pollution—including diesel particles—and lung cancer is tenuous and controversial. He also said that he had “no links of interests with economic actors”

involved in the issue. However, French newspapers revealed last month that Aubier received tens of thousands of euros from petrol firm Total in his capacity as a “medical advisor.” Aubier has denied any conflict of interest. He could face prison time and a fine if convicted of perjury. The Senate's bureau, responsible for settling procedural or disciplinary issues, will decide next month whether to send the case to court. <http://scim.ag/Aubier>

Three Qs

Andrew Witty, who has headed pharma giant GlaxoSmithKline (GSK) since 2008, will retire in March 2017. On 31 March, the Brentford, U.K.-based company announced that it intends to sign agreements about drugs in its cancer portfolio with the “Medicines Patent Pool,” a

A simulation of
"bubbles" of iron-60
atoms surrounding
a supernova.



Supernovae leave their fingerprints on Earth

Have exploding stars affected Earth's climate and the course of evolution? Quite possibly, two studies published this week in *Nature* conclude. Researchers have long wondered whether gamma rays and particles from a nearby supernova could have affected Earth, or even triggered a mass extinction. One solid clue is the isotope iron-60, found in Earth's oceanic crust but almost certainly originating from supernova explosions. One team studied samples of crust from beneath the Atlantic, Pacific, and Indian oceans, and concluded that the iron-60 in the crust must have come from multiple supernova events over the past 10 million years. Meanwhile, by tracing the deposition history of iron-60 on Earth and modeling how it is transported through space by dust grains, the other team determined that the biggest contribution of iron-60 in recent geologic time must have come from two supernovae, each occurring about 300 light-years from Earth; one exploded 1.5 million years ago and the other 2.3 million years ago. Although these cosmic events can't yet be linked to any change in conditions on Earth, let alone a mass extinction, better mapping of iron-60 and modeling of its transport may eventually reveal a smoking gun.

U.N.-backed organization that helps makers of generic drugs license and produce products. Witty, 51, talked with *Science* about his conviction that big pharmas have to be more in step with the needs of the global society.
<http://scim.ag/WittyGSK>

Q: Why are you concerned about your future drugs reaching poor countries?

A: We're trying to ... graduate what we do around intellectual property according to economic maturity of the countries. We don't think you should treat Malawi the same way as you treat Britain.

Q: Are you including cancer immunotherapy products, which are potentially going to be very expensive?

A: They would be included. They not only are likely to be expensive, but they're going to require pretty sophisticated medical systems and technologies. I'm not saying this is going to solve

everything. Some of these things will just be out of reach in some of these countries.

Q: How do you separate making a profit and doing good?

A: You start by trying to do the right thing first, and try to profit maximize second. ... Over the last several years, we've increasingly been prepared to challenge and change our core business model. ... Over time people have followed us. If you look at the fourth quarter of last year, 16.5% of our pharmaceutical sales came from new products, which is the highest for any big pharma. That says you can innovate your business model ... and at the same time do very, very well for the shareholders.

FINDINGS

Rewriting Hep C epidemic history

An analysis of 45,000 genetic sequences of hepatitis C viruses (HCVs) that have infected

BY THE NUMBERS

945

Survival, in days, of a heart transplant from a pig to a baboon, the longest to date due to an immune-suppressing drug therapy (*Nature Communications*).

5%

Decrease in minutes of coverage of climate news on the four major broadcast networks from 2014 to 2015. The drop is mostly due to ABC, which cut climate stories by 59%.

<http://scim.ag/climnewsdrop>

77%

Drop in population of the world's largest primate, Grauer's gorilla, over the past 20 years, leaving fewer than 3800 individuals. The gorilla is found only in the war-torn Democratic Republic of the Congo (Wildlife Conservation Society).

people in North America questions the popular theory that the epidemic exploded largely because injecting drug users shared needles and syringes. Instead, the new study suggests, medical accidents primarily spread the liver-damaging virus, which infects an estimated 4.7 million people in the United States, Mexico, and Canada. The report appeared online 30 March in *The Lancet Infectious Diseases*. A research team led by Jeffrey Joy, an evolutionary biologist at the BC Centre for Excellence in HIV/AIDS in Vancouver, Canada, used the mutation rate of HCV to backdate its history and found that the epidemic took off between 1940 and 1960—about 15 years earlier than the period when injecting drug use dramatically increased in North America. However, the researchers note, until 1950, reuse of glass and metal syringes in hospitals and clinics was commonplace and that sterilization was done manually. Blood transfusions also transmitted HCV until the advent of a test for the virus in 1992.

PHOTO: MICHAEL SCHULREICH



The Angolan military administers yellow fever vaccine at a market in Luanda in February. The disease has since spread far outside the Angolan capital.

INFECTIOUS DISEASES

Yellow fever outbreak triggers vaccine alarm

A major resurgence of the virus in Angola leaves the world empty-handed

By Kai Kupferschmidt, in Dakar

The three people dressed in baby blue plastic suits and goggles form a human conveyor belt for chicken embryos. The first takes a tray of eggs that were injected with a yellow fever vaccine virus, then left to incubate for 4 days, and cuts the top off each egg. The second tweezes the embryos out of the eggs and deposits them in a large bottle. The last person adds some liquid, then blends the embryos into a rich, red broth that contains millions of weakened virus particles.

The end result of this procedure, repeated dozens of times every week at the Pasteur Institute here, is a highly effective vaccine that offers lifelong protection against yellow fever. But the 80-year-old process is decidedly low-tech and hard to scale up—and that's become a problem, because a big yellow fever outbreak that started in December 2015 in Luanda, Angola's capital, has emptied the world's strategic reserves of the vaccine.

The Pasteur Institute, which makes about 10 million doses a year, is one of only four facilities around the world producing yellow fever shots, joining two government-run plants in Russia and Brazil and French vaccine company Sanofi Pasteur. Their combined output has long fallen short of the world's needs, and the Angola outbreak

has worsened the shortfall. Another major outbreak—for instance in Asia, where yellow fever has never gained a foothold—could be impossible to control, says virologist Jack Woodall, a moderator at the Program for Monitoring Emerging Diseases, an online alert system. “I hate being alarmist,” says Woodall, “but this is some-

5,804,475

Number of people vaccinated against yellow fever in the last 2 months in Angola's capital, Luanda.

thing I'm really panicking about.”

The vaccine is the only bulwark against yellow fever, a formidable killer without a cure that is transmitted primarily through the bite of *Aedes aegypti*, also known as the yellow fever mosquito. Most infected people have no symptoms at all; some experience fever, joint pains, and headaches. But roughly 15% progress to a more severe stage in which their eyes and skin turn yellow; they may also bleed from the eyes, nose, and mouth. Up to half of these serious cases are fatal. Although yellow fever is endemic in much of Latin America, Africa bears by far the highest burden. Exact numbers are hard to come by, but a study published in 2014 in

PLOS Medicine estimated that the disease kills 78,000 Africans every year—although many experts felt that number was too high.

Most infections occur in or close to the jungle, where mosquitoes spread the virus primarily between monkeys and occasionally infect a human bystander as well. Urban outbreaks, like the one in Angola, can be far more severe, because mosquitoes can transmit the virus from person to person. “That's when the disease can really take off,” says William Perea, of the World Health Organization's (WHO's) Control of Epidemic Diseases department in Geneva, Switzerland. Angola has seen 490 confirmed cases and 198 deaths so far, but experts say the real toll may be 10 times as high. “We haven't seen an outbreak like this in many years,” Perea says.

A huge vaccination campaign launched in February has already reached 6 million of Luanda's roughly 7.3 million inhabitants. But the disease has since spread to six of the country's 18 provinces, and the global emergency stockpile of 6 million doses is empty. “This is definitely a stressful situation,” says Melissa Malhame of Gavi, the Vaccine Alliance, a Geneva-based public-private partnership that aims to increase immunizations in poor countries.

A ramped-up battle against yellow fever had already strained the global supply of vaccine. Many countries have made the

shot part of their routine vaccination schedules for children, while massive catch-up campaigns were launched to protect entire populations that had never received the vaccine. A United Nations Children's Emergency Fund report last year estimated that the organization needed 42% more vaccine in the next 3 years than is available to it. A 2013 report put global production in 2009 at 75 million doses, up from 30 million in 2000 but well short of the 105 million doses needed that year. The exact annual production today is unknown, but it's probably about 80 million doses, says Tom Monath, a virologist who has studied yellow fever for decades and currently works at New-Link Genetics, a biotech company in Ames, Iowa. To make matters worse, the factory in Dakar is about to shut down for a 5-month renovation.

Things may get better in the long run. Demand for the vaccine should decline in a few years, after countries wrap up their catch-up campaigns. The Pasteur Institute is building a new facility in Diamniadio, about 30 kilometers from Dakar, that could triple production by 2019, and Sanofi Pasteur has built a new facility in France.

In the meantime, WHO has urged Angola to vaccinate only in areas where yellow fever is spreading. But infected travelers have already brought the disease from Angola to three other countries in Africa, including the Democratic Republic of the Congo; if the disease started circulating in its sprawling capital, Kinshasa, that could be catastrophic, Monath says.

"I think all the specialists in my field agree that there is a real and present danger of having a major outbreak of yellow fever that is uncontrollable," adds medical entomologist Paul Reiter of the Pasteur Institute in Paris. "It's a ticking time bomb." One stopgap measure might be to lower the vaccine dose, Monath says; some studies have shown that just one-fifth or one-tenth of the current dose could protect people.

Spread to Asia is the nightmare scenario for yellow fever experts. Angola is home to many Chinese workers, and in at least six cases they have already brought the virus to China. Five of these cases occurred in Beijing, where *A. aegypti* does not occur, so the disease could not spread. But the mosquito is abundant in southern China and elsewhere in Asia—and so are vulnerable people. Oddly, however, yellow fever has never taken off on that continent.

Scientists have come up with various theories to explain that absence—but perhaps Asia has just been unbelievably lucky. "It didn't happen before, but does that mean it is not going to happen now?" Perea asks. "Nobody knows." ■

ECOLOGY

An unhappy peace dividend

With business booming, Colombia is planning to dredge a new shipping lane through surprisingly resilient coral reef

By Lizzie Wade

In 2014, Mateo López-Victoria was hunting for a degraded coral reef off the Colombian coast to test his idea that sponges would invade the struggling habitat. The Varadero sector of Cartagena Bay seemed promising. For 500 years, a canal has discharged wastewater and sediments into that part of the bay. But when López-Victoria dived there, he encountered huge coral colonies. Colleagues have since tallied 30 coral species in the 1-square-kilometer reef, which has 80% coral cover, one of the highest proportions in Colombia. "It turned out to be much more interesting than what I went looking for," López-Victoria says.

Now, having survived centuries of pollution, the reef faces a new threat: an end to Colombia's decades-long guerrilla war.

The prospect of peace is bringing economic development, and a new shipping lane is planned for Cartagena Bay. "They're going to dredge right through [the reef]," says López-Victoria, a marine biologist at Pontifical Xavierian University, Cali, in Colombia. He and others worry that they might run out of time to unlock the secrets of Varadero's resilience, which could help

save embattled reefs around the world. The Varadero reef "should be declared a protected area immediately," says Luis Solórzano, executive director of the Caribbean program at The Nature Conservancy (TNC) in Miami, Florida. So far, no action has been taken to protect it.

For more than 50 years, guerrilla warfare in Colombia retarded development—and inadvertently protected its biodiversity (*Science*, 2 August 2013, p. 450). Now, the government is on the verge of inking a peace deal with its main adversary, the Revolutionary Armed Forces of Colombia, or FARC. "I'm in favor of signing the peace deal so that our country can change," says Valeria Pizarro, a marine biologist at the Ecomares Foundation, an environmental nonprofit in Cali. "But peace is going to have strong environmental repercussions."

Deforestation is the main threat to Colombia's terrestrial ecosystems, experts say. Mining contracts and the spread of agriculture into newly secure areas will affect Colombia's chunk of the Amazon rainforest and habitats cradled between the two chains of the Andes that run through the country. Runoff from development will affect the country's rivers and coastlines as well, marine scientists point out. So far,



Starved of light, Varadero's corals have adopted broad, flat shapes typically seen in deeper waters.

Pizarro says, “The government isn’t paying attention to the scientific community.”

With peace on the horizon, modernizing Cartagena’s port has become a high priority for Colombia’s government and business community. The port is jammed with vessels thanks to rising international trade, and the recently expanded Panama Canal that will soon send supertankers its way. The new shipping lane, which would be 18 meters deep, is meant to ease congestion by providing an alternate route into the bay.

Finding and studying resilient reefs is now in vogue among coral conservationists. Some are investigating corals that have withstood the months-long bleaching event that has decimated reefs worldwide (*Science*, 1 April, p. 15). At TNC, Solórzano’s program grows resilient corals in nurseries and then transplants them to struggling reefs. Other scientists advocate breeding corals like crops to maximize their chances of surviving in warmer and more polluted waters.

Varadero presents an opportunity to study how corals cope with pollution, ship traffic, and sediments—woes threatening many other reefs worldwide. “All the solutions to the environmental changes they’ve been through are in there, in the genomes of these organisms,” says Roberto Iglesias-Prieto, a marine biologist at the National Autonomous University of Mexico, Quintana Roo. For example, sediments in the wastewater flowing into Cartagena Bay block much of the sunlight that would normally filter down to the reef. In response, López-Victoria says, the shallow water corals have adopted broad, flat shapes typically seen in much deeper waters. Iglesias-Prieto would like to study how Varadero corals

“Peace is going to have strong environmental repercussions.”

Valeria Pizarro, Ecomares Foundation

and their microbial communities detect light levels and change shape accordingly.

With Colombia on the brink of change, “construction [of the shipping channel] is perhaps inevitable,” López-Victoria says. “But we can’t permit these projects to happen without keeping in mind their environmental cost.” He and his collaborators are seeking funding to bank samples from Varadero before the reef is gone. “It’s equivalent to saving a great diversity of seeds from our crops,” he says, “so that on the day when they fail, we have some place to go to recover what we’ve lost.” ■



ARCHAEOLOGY

Tallying the losses in Palmyra

After Islamic State retreats from city, talk of restoration

By Zach Zorich

Archaeologists are getting their first look at how a nearly year-long occupation by the group known as the Islamic State (IS) has affected the World Heritage Site of Palmyra in Syria. Government forces retook the historic city late last month, and although satellite images and recent photos show substantial damage to the city’s ancient art and architecture—some of it deliberate—researchers are encouraged that the destruction was not worse. “I’m cautiously optimistic,” says Michael Danti of the American Schools of Oriental Research (ASOR), a scholarly organization based in Boston, which this week released an assessment of the damage.

Officials are already discussing plans to restore damaged sites to their former glory. But some experts disagree about how restoration should proceed, whereas others worry that such talk is premature given that IS still poses a threat to the city and that there is no end in sight to the 5-year-old Syrian conflict. “Things in Palmyra went from frying pan to fire, and now it’s back to frying pan,” Danti says.

Palmyra has long held a special place in Middle Eastern studies. The city, which sits

in central Syria about 200 kilometers northeast of Damascus, reached its cultural peak in the first through third centuries C.E., when it was a Roman empire trading center that attracted Greek, Persian, and Arab merchants. The cultural blending left a distinctive mark, including unique sculptures, tombs, and temples.

IS fighters destroyed many of these cultural treasures after they captured Palmyra in May 2015, and researchers are beginning to tally the losses. Danti says the path of devastation documented by satellite images and local residents reveals the group’s priorities. First, IS fighters destroyed Tadmor prison, a 20th century structure where the Syrian regime jailed political prisoners, perhaps in a bid to curry favor with city residents. Then, the group’s focus became “cultural cleansing,” Danti says. It razed ancient sites that were holy to Islamic groups whose beliefs IS rejects, including the tomb of the Sufi saint Shagaf as well as a number of Sufi and Shia cemeteries and shrines. IS fighters then targeted prominent sites with less direct religious connections, including a massive Roman triumphal arch and a famous statue of a lion in the Palmyra museum that had once adorned a temple of the Semitic goddess al-Lat. The battle to retake the city took an additional toll, with



bombs and artillery shells hitting mosques and other major structures. “A whole landscape has been attacked, not just the World Heritage Site,” Danti says.

Still, the destruction could have been even greater, researchers say. Many important sites appear to have survived, including a military camp and theater dating to Roman times, a historic tax collecting center, and a temple to the Babylonian god Nabu.

Thorough field assessments are not yet possible, because crews are removing thousands of mines and booby-trapped explosive devices left behind by IS group fighters. In the meantime, Danti and his ASOR colleagues have been examining recently released satellite images. They show that at least a dozen Roman-era towerlike tombs, built to house the dead of wealthy families, have been destroyed, according to the forthcoming ASOR report. Five of the stone tombs were destroyed within the past 5 months, the images suggest.

Photos taken by journalists show that the museum has been ransacked, with pieces of statues littering the floor. Curators moved much of the museum’s collection to Damascus before Palmyra became a battleground, according to Syria’s Directorate-General for Antiquities and Museums (DGAM), but left behind some larger objects.

Specialists should be able to reconstruct the shattered sculptures, given enough time and money, says Maamoun Abdulkarim, the head of DGAM in Damascus. He is already in discussions with United Nations Educational, Scientific and Cultural Organization officials about plans for bringing back the

Islamic State group fighters demolished much of Palmyra’s Temple of Bel, which dates to the second century C.E., in August 2015 (left). Above, the temple in 2010.

larger sites. It could take just 5 years to restore Palmyra, he estimates, given sufficient money and help from the international community.

Some specialists, however, are skeptical of that timeline and predict restoration will pose complex problems, including how any reconstruction should reflect Palmyra’s recent violent history. “It’s not just a heritage site anymore. It’s a war zone, it’s a grave, it’s a place where people were executed,” says Allison Cuneo of ASOR. She notes that IS fighters used a Roman amphitheater to stage the executions of at least 25 men. “That theater where they killed all those soldiers is a place where they were holding plays and ballets,” she says. “Now, people are going to remember it as the place where their son was killed.”

Others worry that talk of restoration is premature. Amr Al Azm, a Syrian archaeologist now at Shawnee State University in Portsmouth, Ohio, notes that the Russian military forces that helped the Syrian government retake Palmyra are slowly withdrawing from the country. And a cease-fire that provided the government with a tactical advantage is coming to an end. Another shift in the balance of power, he worries, could put Palmyra’s treasures back in the hands of IS. ■

Zach Zorich is a journalist based in Fort Collins, Colorado.

SOCIAL SCIENCE

Ironic coda to fraudulent study of bias

Research by whistleblowers who exposed gay canvassing study finds same effect

By John Bohannon

Last summer, while media clamored for him to comment on a scientific scandal he had helped reveal, David Broockman was keeping an explosive secret of his own. Just months earlier, he and Joshua Kalla, political scientists now at Stanford University in Palo Alto, California, and the University of California (UC), Berkeley, respectively, had revealed a study published by *Science* in 2014 as likely resting completely on fake data. Now, however, Broockman’s own work was confirming that the effect claimed by the fraudulent study was real after all.

The study asserted that a short interview by a gay canvasser, if done right, can powerfully reduce people’s prejudices, specifically about same-sex marriage, a “finding” that stunned social scientists. But Broockman and Kalla found discrepancies in the paper, and its lead author, political science graduate student Michael LaCour, never produced the raw data to address them. Meanwhile, the two whistleblowers had their own study underway to test the same canvassing technique with another hot-button topic: transgender people. “Journalists were still calling us about LaCour when the first wave of data were coming in from our study,” Broockman says. “It was terrifying.”

In one of the strangest twists in social science history, their study, published on p. 220, shows that the canvassing strategy really can influence biases. “The data are solid and the analysis convincing,” says Gabriel Lenz, a political scientist at UC Berkeley who was asked by the funders of the study to verify that the data were truly collected. The effect is “so large and enduring,” he says, “that many researchers will be skeptical.”

The new study and the retracted one both focus on a persuasion technique pioneered by the Los Angeles Lesbian, Gay, Bisexual, and Transgender (LGBT) Center in California, whose canvassers have conducted more than 13,000 face-to-face interviews over its

nearly 50-year history. “Prejudice against transgender and gender-nonconforming people is a terrible daily reality,” says the center’s director, David Fleischer. So the canvassers aim not just to survey existing prejudices or spread awareness, but to permanently change people’s minds.

They are up against decades of research that have produced little evidence that such biases can be altered, says Elizabeth Paluck, a political scientist at Princeton University. And because attitudes toward transgender people often involve deeply held beliefs and strong emotions, she says, “many scholars would have pegged transgender prejudice as more persistent than others.” That was what made the results of LaCour’s now retracted study all the more amazing.

After trying many different persuasion techniques over the years, the LGBT Center has its canvassers follow one called

For example, LaCour reported that 92% of the people who took part were interviewed at work, “but not even that many people have jobs,” Broockman says. Also, the response rate seemed unbelievably high.

With just 2 weeks to go before they launched their own study, Broockman recalls, “we were in a panic.” So they reached out to LaCour’s co-author, Donald Green, a political scientist at Columbia University who was a mentor to both Broockman and Kalla. Soon after he learned of their concerns, Green started the process that led to his voluntary retraction of the paper. (LaCour disputes that the data were fraudulent and did not consent to the retraction.)

For their version of the study, Broockman and Kalla sent 56 canvassers—some transgender, others not—to knock on the doors of 501 people living in Miami. As a control, some of the interviews focused not on transgender discrimination, but on recycling. In all cases, the 10-minute interview included a survey before and after to measure people’s attitudes regarding transgender people, as well as follow-ups ranging up to 3 months later.

The effect was as powerful as LaCour’s supposed results: The canvassing technique virtually erased the transgender prejudices of about one in 10 people, and the change lasted at least 3 months. However, Broockman and

Kalla found that the interviews reduced prejudice regardless of the gender status of the canvasser, in contrast to the retracted study, which suggested that the interviewer had to be a representative of the victimized population for the change to stick.

“The findings are compelling and it will be important to see how generalizable they are in future studies,” says political psychologist Diana Mutz of the University of Pennsylvania. Opinions on the relatively new topic of transgender people, she notes, “may not be fully crystallized, thus potentially making them easier to persuade on this issue than other well-established controversies such as gay marriage.”

Green says he is pleased that the LGBT Center’s approach has been vindicated. The center “suffered a terrible blow when LaCour’s surveys turned out to be phony, as the center’s outreach efforts were written off by many as naïve,” Green says. “Now, the center has a proper scholarly evaluation of its innovative and important work.” ■

INDUSTRIAL ESPIONAGE

3D printers vulnerable to spying

Design information can be pilfered from the sounds a printer makes

By Mara Hvistendahl

From online shopping to social media, the power and convenience of digital technologies often come with a cost in security. Three-dimensional printing, the versatile technology that can churn out everything from engine parts to prosthetic limbs, appears to be no exception. By building objects layer by layer, rather than chiseling away at materials and assembling parts, 3D printers can make individualized products with minimal waste. But the signals that a printer sheds as it goes through its digitally controlled paces render it vulnerable to attacks, scientists have discovered.

A simple audio recording—possibly even one made by a smartphone—can be enough to reverse-engineer a 3D-printed object, a new paper shows. From the sounds the device emits while printing, an attacker can recreate the source code that contains the shape of the 3D-printed object. “Industries spend millions of dollars to create IP [intellectual property], and you can basically steal it by listening to the machine,” says Mohammad Al Faruque, a researcher in the emerging field of cyber-physical systems at the University of California, Irvine. He and colleagues will present their research on 11 April at the International Conference on Cyber-Physical Systems in Vienna.

Listening in on sound waves to steal IP is a “unique” idea, says Chris Williams, a mechanical engineer at Virginia Polytechnic Institute and State University in Blacksburg who was not involved in the research. But what’s “really exciting,” he says, is that the paper not only exposes a security issue—it implicitly suggests a possible fix for other methods of IP theft.

Since the 1970s, scientists have known that signals emitted by industrial machines—so-called side channels—contain useful information. In digital manufacturing, the sound of machinery is often monitored for quality control. “People are already using acoustics as a means of listening for



Rallies have highlighted discrimination against transgender people.

“analogic perspective taking.” By inviting someone to discuss an experience in which that person was perceived as different and treated unfairly, a canvasser tries to generate sympathy for the suffering of another group—such as gay or transgender people. “We knew from our own periodic attempts at self-measurement that we appeared to be achieving strong, lasting results,” Fleischer says, but the group wanted more proof.

So the LGBT Center reached out to academic researchers to rigorously test the technique. Unfortunately for them, the first one they worked with was dishonest. The scandal was “like a big punch to our collective gut,” Fleischer says. But he knew that Broockman and Kalla were evaluating the same canvassing technique in Miami, Florida, with funding from the Gill Foundation, a Denver-based nonprofit that promotes equal rights for LGBT people.

That is why the pair examined LaCour’s results so carefully. The closer they looked, the more the study just didn’t make sense.



Simple spycraft may be able to intercept design details of 3D-printed objects such as this soft tissue prosthesis.

defects, like a tool breaking,” Williams says.

But side channel emissions can also provide a window into the IP at stake, Al Faruque and his colleagues showed. Using a Printbot 3D printer, the researchers printed several sizes and shapes of objects—such as a square, a triangle, and a mock house key—while recording the hums and beeps made by the machine. Then they tested how well the source code for each could be reconstructed from recordings. Borrowing methods from speech pattern recognition, they found that a computer could “learn” an object’s code with an average accuracy of 78%. The accuracy with a key, the most complex shape tested, was 92%. (The square and triangle were smaller, which made their designs harder to recreate.)

Co-author Arquimedes Canedo, a cyber-physical systems researcher at Siemens Corporate Technology in Princeton, New Jersey, says he was surprised at “how quickly we got to the reported results, and how quickly we have been improving the accuracy.” The researchers placed the recorder at a specific angle 30 centimeters from the printer. “Whether the same accuracy can be achieved at greater distances, behind walls, different angles, [and] in noisy environments” is fodder for future research, Canedo says.

The new method won’t tell an eavesdropper about the printer’s temperature or other settings that affect the material of a 3D-printed object, says Mark Yampolskiy, a cyber-physical systems researcher at the University of South Alabama in Mobile. When 3D printing objects like jet engine turbines, he says, printer settings are crucial because they indirectly reveal information such as “whether your jet is supposed

to operate at a particular rotation [speed].”

Al Faruque notes, however, that “attack models can always be enhanced” by targeting additional side channels like thermal profiles and electromagnetic radiation. Expensive IP “can be a big motivation for attackers to spend money on powerful infrastructure,” he says.

3D printing is ripe for piracy because of widespread home use and an enthusiast design-sharing culture, Williams says: “You can buy one at Best Buy and have it on your desk and be printing within hours.” In 2013, Gartner, a consulting firm in Stamford, Connecticut, estimated that 3D printing of pirated designs will result in annual IP losses of \$100 billion by 2018.

The outlook isn’t entirely bleak, Al Faruque notes: “The flip side is that this information can also be used for detection.” The team is now attempting to prove that analog emissions like sound waves subtly change when a machine’s software is under attack. (They declined to provide additional details.) If that research pans out, companies would be able to detect certain types of attacks on IP by checking for anomalies in printer sounds.

The team is also exploring potential countermeasures, such as inserting white noise into the printing process. Williams and colleagues, meanwhile, have designed a method of marking legitimate 3D-printed products with unique signatures of quantum dots—particles that are visible in UV light. “You’d have to destroy the product to get rid of that tag,” Williams says. But Al Faruque warns that more sophisticated devices will only lead to more imaginative attacks. ■

FEATURES

ONLY THE STRONG SURVIVE

Politics and budget pressure reshape science in Putin's Russia

By Richard Stone, in Moscow

Nikolai Kolachevsky opens a curio cabinet in his office and carefully removes a relic of Russia's glorious scientific past: a small glass case containing the exquisite instrumentation crafted by physicist Pyotr Lebedev to carry out his landmark experiments on light in 1899. Using tiny vanes strung with wafers of platinum, aluminum, nickel, and mica, Lebedev was the first to measure the minuscule pressure exerted by light. "It was remarkable work," says Kolachevsky, who leads the Russian Academy of Sciences's (RAS's) P.N. Lebedev Physical Institute (LPI) here.

Kolachevsky hopes to foster similarly elegant work at LPI—one of Russia's oldest and best-known scientific institutes. The institute's latest foray into the frontiers of physics is a center for high-temperature superconductivity named after Vitaly Ginzburg, the Russian who shared the 2003 physics Nobel for theoretical work on superconductivity and died in 2009. "It was his dream to create a room-temperature superconductor," Kolachevsky says. Starting in 2018, the new center, 100 scientists strong, will take up that quest.

LPI may be a crown jewel among RAS's 826 institutes, but its future is hardly secure. It was one of several vaunted institutes left off a list of Russia's 150 top performers, compiled at the end of last year by the science ministry based on criteria such as publications and funding. Critics contend that the ranking is tainted: Since the Soviet

collapse in 1991, the ministry has sought to strengthen science in Russia's universities, sometimes at the academy's expense. Kolachevsky, who at 44 is RAS's youngest institute director, says only that LPI's omission "was quite painful." He's making contingency plans for budget cuts.

For institutes lacking the luster of LPI, omission from the list bodes much worse. Russian President Vladimir Putin has signaled that a day of reckoning has arrived for Russian science. Presiding over a meeting

"It was a message for all of us," says Putin's science aide, Andrei Fursenko. "It's impossible to subsidize organizations over the long term that do not produce any scientific output. That is bad for the state and for science." RAS is not alone in feeling the chill: Scores of institutes outside RAS are expected to be axed or merged. (Altogether Russia has about 1700 state scientific institutes.) At the same time, the government is showering riches on a few research centers with sterling track records or more political clout, notably the Kurchatov Institute here, a physics bastion that has absorbed several former RAS institutes in recent years.

Everyone agrees that Russian science is in dire need of reform. Scientific productivity is waning. Russia last year published roughly the same number of papers as Iran, a rising science power, says RAS President Vladimir Fortov, here. A roster of the top 1500 most-cited scientists, he says, includes only two living in Russia. The bleak picture is unsurprising, given a massive brain drain after the collapse of the Soviet Union and the

penury endured by scientists who stayed behind. RAS's annual budget, roughly \$1.2 billion, "is nothing!" Kolachevsky exclaims. "We are blamed regularly for not having enough publications, and not having modern labs," he adds. "It's like you don't feed a person for 20 years and say, 'You're pale and your eyes are not shiny. Why aren't you working hard?'"

Yet the venerable academy, founded in 1724 by Peter the Great, is not going down without a fight. At the council meeting,



At January's science council meeting, Putin called for triage of research institutes.

of the Presidential Council for Science and Education in the Kremlin on 21 January, Putin noted that the 150 institutes on the list account for "a vast majority" of the nation's scientific output: some 70% of all patents issued in Russia and 80% of "highly cited works." Putin then asked the assembled science elders: "What about the others? How are they doing?" before declaring that when setting priorities, resources should be allocated to "strong research teams that are capable of creating breakthrough technology."



Soyuz launches like this
one last month sustain
the International Space
Station. Now, Russian
space science is showing
new strength.

Fortov, a physicist, summoned the moxie to challenge Putin, pointing out that oases of good science exist in weak institutes. He and other prominent voices argue vehemently against a winnowing that could shutter 90% of RAS's institutes. Many outside the science centers of Moscow and St. Petersburg—from Dagestan and Tatarstan to Bashkiria and Siberia—would be axed, says Robert Nigmatulin, director of the P.P. Shirshov Institute of Oceanology here. “Science unites us,” he says. “Eliminating institutes will ... kill the country itself by destroying the ties connecting the republics.”

Russia's science leaders say researchers who lose their jobs in the coming months—the number could be in the thousands, sources say—will not simply be tossed out on the street. “I assure you that will not happen,” Fursenko says. Some would go into early retirement, whereas others may receive severance pay or retraining. Putin's overriding desire, Fursenko says, is “to minimize apprehension.” But even those who survive the culling will have good reason to be apprehensive. “Let's say they decide to minimize the number of institutes to 150. In 5 years it will be 100. In another 5 years, 50,” Nigmatulin says. “This action will destroy Russia.”

DURING THE COLD WAR, Soviet science provoked awe, not pity. The 1957 Soviet launch of Sputnik, the first satellite, was a watershed moment for science in both superpowers. U.S. President Dwight Eisenhower at first downplayed the Soviet achievement, but it compelled him to bolster U.S. education in math and science and create NASA. Those were also glory years for RAS. From 1956 to 1978, a dozen academy scientists won Nobel Prizes for work such as the discovery of Cherenkov radiation and pioneering research on lasers and in low-temperature physics.

After the Soviet collapse, scientists left in droves for positions in Europe and the United States. Science withered under Boris Yeltsin, Russia's president from 1991 to 1999. When physicist Mikhail Kovalchuk was appointed director of the Institute of Crystallography here in April 1998, “the first letter on my desk was an offer to buy secondhand goods from the United States for \$3 per kilogram. But we could hardly afford food or water.” That same year, Albert Galeev, then-director of the Space Research Institute (IKI) here, visited Prime Minister Sergey Kiriyenko to plead for space research funding. “Kiriyenko bluntly told him that space was not Russia's first priority. Nor even

its second,” says plasma physicist Roald Sagdeev, a former IKI director who is now at the University of Maryland, College Park.

About a decade ago, Russian science started pulling out of its death spiral. Scientists received a series of pay hikes, and institutes revved up plans for big science projects. But desultory efforts to reform the increasingly hidebound RAS sputtered. In the late 2000s, Fursenko, as science minister, set out to bolster research in Russia's universities. Part of his aim, he says, was to give the academy some competition—and prevent it from “getting worse and worse.”

Fed up with RAS's resistance, the Russian government in June 2013 pushed through a law compelling it to merge with the medicine and agriculture academies. The law also led to the creation of a new agency that owns all RAS property and controls most of its budget. Russian scientists braced for a fire sale. “The worst expectation was that a large part of the property belonging to the academy would be lost to science,” says Valery Rubakov, a physicist at the Institute for Nuclear Research here. But Fortov, elected RAS president just a few weeks before the law was passed, won a 2-year moratorium on the sale of institute property (extended for one more year), preserving an uneasy peace.

Global tensions rile experimental university

In 2011, the Massachusetts Institute of Technology (MIT) in Cambridge surprised many by gambling on a joint venture with Russia: a U.S.-style research university. Russian scientists looked on with envy as the federal government poured tens of millions of dollars into the Skolkovo Institute of Technology (Skoltech), part of a high-tech park on Moscow's outskirts that was a pet project of former Russian President Dmitry Medvedev. “MIT gave credibility to the whole enterprise,” says Anton Berns, a molecular biologist at the Netherlands Cancer Institute in Amsterdam who came to Moscow in 2013 to launch a new stem cell center.

Skoltech is still seeking to live up to its promise. The hope was that teaming up with

MIT would set it apart from top-tier Russian universities, such as Moscow State and the Moscow Institute of Physics and Technology, by focusing on innovation and by knitting research more deeply into higher education, mending a Soviet-era rift. “Nothing was imposed on us,” says Edward Crawley, a former MIT aeronautics professor who just wrapped

up a 5-year term as Skoltech's first president. “We had fairly broad latitude in shaping the culture of the project.”

But success is hard to judge. “Nobody defined Skoltech's main mission,” says Andrei Fursenko, science adviser to Russian President Vladimir Putin in Moscow. Meanwhile, Russia's relations with the West have deteriorated, hampering Skoltech's efforts to recruit non-Russian faculty, and low oil prices and the ruble's decline have hobbled its economy, pinching funding.

The Russian-funded Skolkovo Foundation, which runs the university and a technology incubator, is sinking \$75 million into Skoltech this year, much of which is for construction of a new

campus slated to open in the coming academic year for its 315 students and 60 professors. But the university is expected to fall well short of aspirations to ramp up to 1200 students and 200 professors by 2020. “We need more diverse funding streams,” Crawley says.

A few non-Russian faculty pulled up stakes early, including Berns, who left Russia halfway into a 3-year commitment. Berns says he intended to stick it out, even though his group would wait weeks for reagents and instruments that arrive quickly in countries with more permissive customs regulations. But his views shifted after Russia annexed Crimea and supported separatists in Ukraine. The “trigger” for his departure, he says, was the July 2014 downing of Malaysian Airlines flight 17 over eastern Ukraine. “One of my colleagues was on that flight.”

Skoltech remains a beacon



PHOTO: SK.RU

Now, the détente is breaking down, as Russia's economic malaise forces the government to cut spending. Over the past 2 years the ruble has lost half of its exchange value against the dollar, in part because of plummeting prices for the oil that supplies a big share of Russia's budget.

Besides hastening RAS's judgment day, the economic straits cast a shadow on a budding science revival. Russian scientists had a moment of celebration in December 2015, when the Joint Institute for Nuclear Research in Dubna, collaborating with three U.S. national laboratories, announced the discovery of three new elements—115, 117, and 118. But a \$50 million expansion of a neutrino detector at the bottom of Lake Baikal in Siberia, originally slated for completion in 2018, will be delayed at least 2 or 3 years, says Rubakov, whose institute leads the project. Meanwhile, IKI is assessing how cuts to Russia's space budget may crimp an ambitious set of science missions, including Russia's return to the moon after 4 decades (see p. 138).

Compounding the woes are European and U.S. sanctions imposed after Russia's March



Budget woes have slowed upgrades to a neutrino detector at Lake Baikal.

2014 annexation of Crimea (see p. 140). Putin has said that the sanctions, which limit exports to Russia of instruments or materials with potential military uses, could “possibly play into [Russia’s] hands” by forcing scientists to be more innovative. Still, frostier relations with the West have hurt a young university seeking to import Western talent: Skolkovo Institute of Science and Technology, or Skoltech, on Moscow’s outskirts, a joint venture with the

Massachusetts Institute of Technology in Cambridge (see below).

A FEW OF RUSSIA’S hallowed science institutes are thriving, thanks to political savvy as well as strong science. One is the former home of the Soviet atomic bomb effort, the Kurchatov Institute, where Kovalchuk is now the president. He has a knack for pleasing the government while heaping criticism on the academy. “I always tell everything on my mind,” Kovalchuk says. “And I never regret what I say.” One of his abiding criticisms of the academy is its sense of entitlement, which he sees as a vestige of the Soviet era, when RAS had the status of a min-

istry. For years after the Soviet collapse, he says, “if the government asked the academy, ‘How are you spending your money?’ the academy would respond, ‘We do science, basic science.’” He laughs. “The academy is full of egg-headed people, like me.”

The antagonism between Kovalchuk and conservative elements in RAS came to a head in 2008, when Kovalchuk, then an RAS vice president, was denied a promotion to full academician. In early 2013, when leg-



for the Russian diaspora: Returnees comprise nearly half the faculty. Albert Nasibulin, for one, found a refuge there in 2014 from an increasingly tense life in Finland. The specialist on carbon nanotubes says his work was thriving at Aalto University School of Science in Helsinki, but he couldn't stand what he saw as rising anti-Russian sentiment in Finland. “I was really pissed off. There was only bad news about Russia in the media.” Natalia Berloff, a

mathematical physicist at the University of Cambridge in the United Kingdom, didn't face such difficulties there, but nonetheless says her tenure at Skoltech was a very good move. “I pushed Skoltech to build labs in my research area,” superfluid turbulence, which gave her “a brilliant opportunity to work closely with experimentalists.”

To Nasibulin, what sets Skoltech apart is its emphasis on entrepreneurship. He and his students are always

scouting for findings that can be spun off into companies. In that sense, he says, “Students think differently here.”

The government's gamble on a Western model will pay off, insists Skoltech's new president, Alexander Kuleshov. He took the reins in February and soon persuaded Igor Krichever, a prominent mathematician at Columbia University, to move to Skoltech next month to head a Center for Advanced Studies. “The state gave Skoltech a lot, and we have to

Skoltech's campus, under new president Alexander Kuleshov (left), will open next academic year.

use that to not only produce a small number of elite graduates,” Kuleshov says. “The authorities understand very well that Skoltech is helping to reconstruct Russia's higher education system.”

“Let's be objective,” says Fursenko, who acknowledges that he was not enamored at first with Skoltech. “It's a work in progress.” —Richard Stone

isolation to reform RAS first appeared in the Duma, the lower house of parliament, many observers saw it as Kovalchuk's revenge. His brother, Yuri, is a prominent banker and friend of Putin's, and Kovalchuk's critics allege that he has exploited that connection to undermine the academy and further his own career. "Kurchatov has a highly privileged position because of Kovalchuk's family connections," says one academician who requested anonymity. "Putin goes to his institute to drink tea with him. Problems get solved very quickly and smoothly."

Kovalchuk says Putin has only visited his institute twice; the last time was 7 years ago. "The Kovalchuks do not have any connections to Putin," he insists. He admits he was stung by the election failure, but insists he does not hold a grudge. Rejection was liberating, he says, as it allowed him to focus on strengthening the Kurchatov Institute. "I came here and built a completely new life," he says.

He has, in fact, built an empire. The Kurchatov Institute now spearheads Russia's nanotechnology research, has a 1.5-petaflop supercomputer used primarily for protein modeling, and is upping the number of beam lines on its synchrotron from 15 to 21. Its 5000-plus scientists work in areas such as protein crystallography, nanoparticles for drug delivery, and high-temperature superconductivity. "It's much easier to explain what we are not doing," Kovalchuk says.

Kurchatov has a generous line item in the federal budget—about \$230 million—and Kovalchuk says it earns about the same amount by building nuclear power components, for example, and manufacturing stents and other biomedical devices. In 2010, the Duma passed a law subsuming three institutes, including two from RAS, under the Kurchatov Institute. One, the Petersburg Nuclear Physics Institute in Gatchina, is now racing to complete work on a 100-megawatt nuclear research reactor that will generate high-energy neutrons for research. Not satiated, the Kurchatov Institute in February absorbed two more institutes, specializing in chemistry and materials science.

That expansion stands in vivid contrast to RAS's impending doom. To Kovalchuk and others, it is merely survival of the fittest. "If you have a factory that has died, but you keep giving them money, you prolong a terrible process. It's much easier, and more honest, to close ailing institutes," he says. Fursenko agrees. "With limited resources, 150 strong institutes is not a bad outcome," he says, adding with a nod to philosopher Adam Smith: "To create wealth in a country, sometimes you have to ruin a lot." ■



POISED FOR LIFTOFF

After 2 decades adrift, Russia's space scientists plan an ambitious set of missions

By Richard Stone, in Moscow

When Russia's Mars-96 exploration mission broke apart after launch in November 1996, the loss cast a pall over Russian space science. "We were barely functioning. There was this feeling of uselessness in the air," says Lev Zelenyi, director of the Institute of Space Research (IKI) here. Now, Russia is hoping to dispel that pall with its biggest slate of lunar and planetary missions since the early 1970s. But budget cuts are threatening to drag the nation's space science revival back to Earth.

In January, the Russian government approved a 10-year plan crafted by Russia's space agency, RosCosmos, covering everything from contributions to the International Space Station to weather and navigation satellites and human space exploration. About 15% of the spending would go to "basic physics in space," says Zelenyi, a plasma physicist. But the plan is consider-

ably leaner than expected. With the government's coffers squeezed by low oil prices, RosCosmos has had to slash its budget for the 10-year plan to 1.4 trillion rubles (\$20.5 billion), down from the 3.4 trillion rubles the agency asked for a year ago.

Nonscience parts of the space program have borne the brunt of the cuts, but a bevy of science missions are also at risk, including the resurrection of Russia's lunar program. Russia hasn't been back to the moon since the space race with the United States a half-century ago. The Soviets scored early with the Luna-1 mission—the first unmanned probe to orbit the moon, in 1958—and Luna-2, which became the first spacecraft to land on the moon in 1959. "It was a really great time for our scientists, when we were competing with America," Zelenyi says. But after U.S. astronauts won the race to the moon, the wildly expensive U.S. and Soviet programs both hit stiff headwinds. The last Soviet mission from that period was Luna-24 in 1976.

Russia's renewed interest in the moon



came after a Russian instrument hitched a ride in 2009 with NASA's Lunar Reconnaissance Orbiter. The instrument, a neutron detector, spotted pockets of subsurface water ice. Russia's leadership had rekindled dreams of putting cosmonauts on the moon—and here was a potential source of water. IKI now has five lunar missions planned from 2018 to 2025, starting with Luna-25, a spacecraft that would land near the moon's south pole. The European Space Agency (ESA) will take part in the first three missions. A highlight is a drill it's designing for Luna-27, which would penetrate a meter into the regolith—the surface layer of dust and rock debris—to take samples. “We don't know if the regolith is soft or hard. If it's saturated with ice, it could be like drilling into concrete,” says James Carpenter, ESA's lead scientist on Luna in Noordwijk, the Netherlands.

Some researchers are unimpressed with that science plan. “All that was done

before—in the 1970s,” scoffs one Russian scientist. Carpenter disagrees. “The moon is not old hat,” he says. All lunar samples have come from a region that's “not representative of the whole. If you want to understand all the science that has come before, you have to go to new places and take samples.”

Humans won't follow for a while. Russia's budget woes will slow the human exploration program beyond the first mission's stated target of 2025, Zelenyi says. But he denies rumors that Luna-25 will be delayed.

Two other cornerstones of the Russian space revival are Mars and astrophysics. Phobos-Grunt, Russia's next attempt to reach the Red Planet after Mars-96, brought back bad memories when it broke up after launch in 2011. Like Mars-96, it ended in a fiery crash in the Pacific Ocean—the subject of “jokes mixed with tears,” Zelenyi says. But Russia is teaming up with Europe on ExoMars, a two-spacecraft mission. The first probe, designed

Soyuz flights to the space station bring U.S. dollars, but a budget squeeze is hitting space science.

to sniff for methane, was launched last month and is now en route to Mars, salving some of the sting of the earlier failures. And IKI and NASA are in early discussions on a possible joint mission to Venus after 2025.

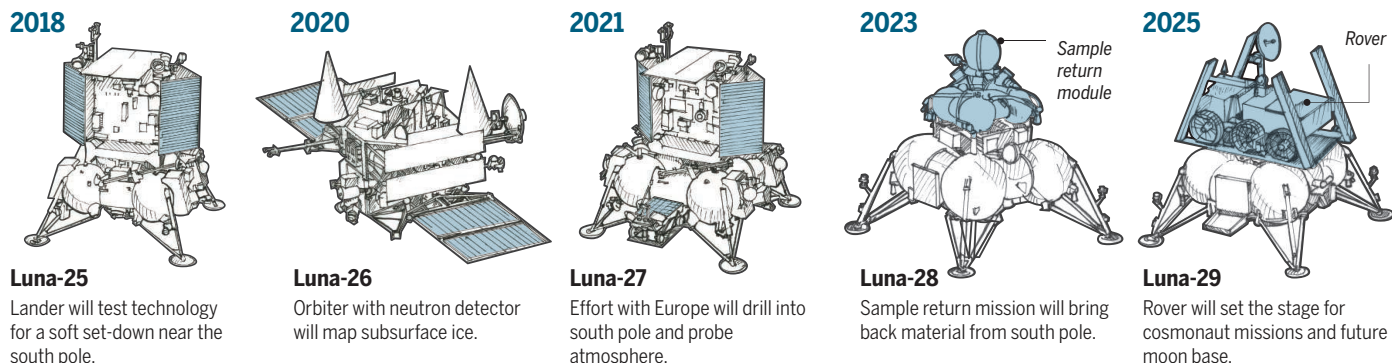
Funds permitting, Russian astrophysics is poised for revival as well. On deck is SPEKTR-RG, a pair of x-ray telescopes that would map x-ray sources such as black holes and neutron stars. First conceived 25 years ago, the long-delayed project, now a joint effort with Germany, was revised twice. It's become even more important to astronomers worldwide after last week's possible loss of Japan's x-ray telescope. “We found a niche, and there will be new physics,” Zelenyi promises. Launch is slated for September 2017, but that may slip, he says.

After that will come Gamma-400, “one of most ambitious projects in the world in next 10 years,” declares Nikolai Kolachevsky, director of the P.N. Lebedev Physical Institute (LPI) here. LPI is taking the lead on the gamma-ray telescope, slated for launch in 2022. Gamma-400 aims to probe the nature of dark matter and the origins of extragalactic cosmic rays, and will search for high-energy gamma-ray bursts. Along with technical hurdles and budget worries, the mission faces the impact of international sanctions imposed on Russia for annexing Crimea. Components that also have military uses, such as equipment for protecting the spacecraft from radiation, now are difficult to procure, Kolachevsky says.

Budget realities may yet force some missions onto the back burner. But for the first time since the Soviet breakup, Zelenyi says, Russian space scientists can look ahead with confidence. “Even though scientists want to have much more than the country can afford,” he says, “the next decade will be quite busy for us.” ■

Russia's giant leap

After a 40-year drought, Russia's looking to get back into the lunar game with five missions over 8 years starting in 2018.



OUT IN THE COLD

Russia's takeover of Crimea has isolated scientists in the strategic peninsula

By **Richard Stone**, in Sevastopol

In 2009, the Marine Hydrophysical Institute (MHI) here scored a coup: It became the only organization outside the European Union picked as a forecasting center in Europe's MyOcean program.

Perched on a bluff in this picturesque port, MHI collected data on everything from currents and salinity to wind speeds and chlorophyll levels in the Black Sea. Then on 1 October 2014, as singularly as MHI was welcomed into MyOcean, it was booted out again. "European financing for us stopped," says MHI Deputy Director Alexander Kubryakov. "Now, we work on pure enthusiasm."

The reason for MHI's ouster: Russia's annexation of the Crimean Peninsula in March 2014. Just six countries, including North Korea, Syria, and Venezuela, now recognize Crimea as part of Russia. Most of the world still considers it part of Ukraine. As the European Union and the United States imposed escalating sanctions on Russia, science collaborations with institutions in Crimea foundered. Researchers here holding Russian passports now face huge hurdles when seeking visas to the West. "Crimea became a kind of black hole on the Earth," says Pavel Gol'din, a zoologist who in 2014 decamped to Kyiv, Ukraine's capital, from Taurida National V.I. Vernadsky University (TNU) in Simferopol.

Shunned by the West, Kubryakov and others who stayed put have received a warm embrace from Russia. Five Crimean institutes are now part of the Russian Academy of Sciences (RAS). Scientists at MHI and the A.O. Kovalevsky Institute of Marine Biological Research (IMBR), also in Sevastopol, are joining RAS-led marine expeditions. The Crimean Astrophysical Observatory in Nauchny, meanwhile, got Russian funding

this year to purchase a pair of 70-centimeter telescopes that will search for dangerous near-Earth asteroids. Crimean scientists with an affinity for Russia profess that they are content. "We came back home," says Elena Nevrova, a phytoplankton specialist at IMBR.

Ukraine may have lost control of Crimea, but it won't relinquish it. The government has made it illegal for Ukrainians and foreigners alike to travel to the peninsula via Russia. In October 2015, the Ukrainian Academy of Sciences told its scientists to suspend all cooperation with colleagues in "temporarily occupied" Crimea. And last month, displaced professors from TNU opened a campus in Kyiv that will offer online courses for students in Crimea. TNU-in-exile, says rector Volodymyr Kazarin, who headed the

Russian literature department in Simferopol, will develop "real tools for the deoccupation of Crimea" by training the next generation of Crimean leaders.

FAMED FOR ITS vineyards and Black Sea resorts, Crimea for millennia was a crossroads of East and West.

The peninsula—the size of Maryland—was a hub for trade between Asia's steppes and the Mediterranean Sea. But the ancient crossroads has often been a focus of conflict. In the 1850s the Crimean War pitched Russia against the Ottomans, the United Kingdom, and France, while in 1944 Soviet leader Joseph Stalin ordered the deportation of all 240,000 Crimean Tatars, an ethnic minority, to Central Asia.

Following the Soviet collapse in 1991, Russia kept its Black Sea fleet in Sevastopol, leasing the port from Ukraine. But after protests in Kyiv that began in November 2013 drove out Ukraine's pro-Russian president a few months later, Russia seized the strategic peninsula. "Everyone expected the Ukrainian army would defend Crimea and

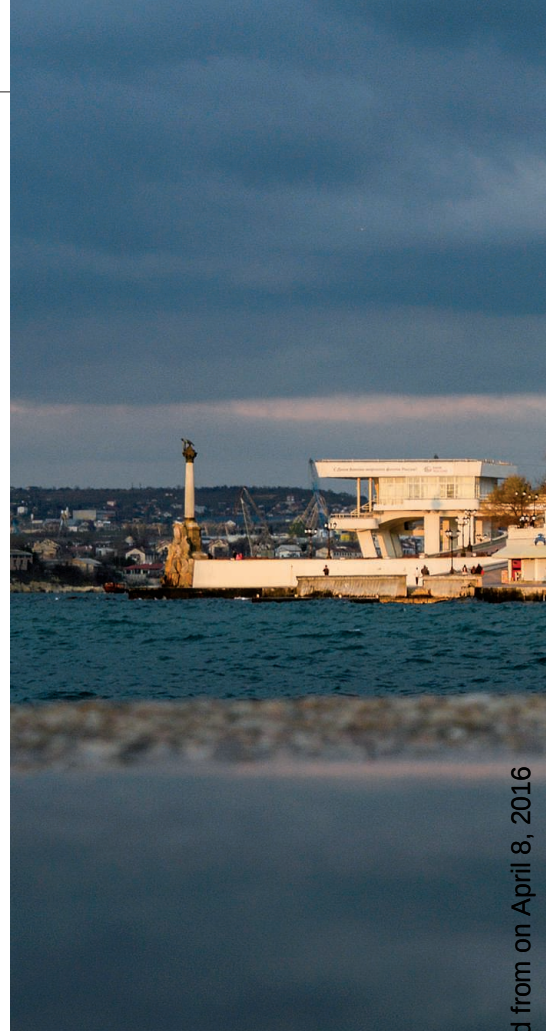
"Crimea became a kind of black hole on the Earth."

Pavel Gol'din,
Institute of Zoology, Kyiv

there would be a lot of bloodshed," Gol'din says. Pro-Russian men in Crimea, including many young scientists, mobilized into regiments. "It was scary. We didn't have any weapons," says one IMBR scientist who requested anonymity because he says "Ukrainian fascists" are targeting him.

Now, billboards across the peninsula show the smiling visage of Russian President Vladimir Putin and three words in Cyrillic declaring: "Crimea. Russia. Forever." "Some institutes adopted the Russian system quickly, others tried to resist," says Gol'din, who's now at the Institute of Zoology in Kyiv. The Crimean Astrophysical Observatory was in the former camp. When European colleagues emailed the observatory's Konstantin Grankin, saying they were sorry about what happened, "I didn't know how to answer them," Grankin says. "I just sent them photos of us enjoying Maslenitsa," a pagan celebration in March to welcome spring featuring burning scarecrows and Russian blini pancakes.

But the takeover rent Crimea's scientific community, sparking an exodus. TNU, Crimea's premier university, was decimated; Russia has merged it with several others to form a new Crimean Federal University in Simferopol. At the Institute of Archaeology,



Downloaded from on April 8, 2016

PHOTO: ALEXANDER ZHELEZNYAK

Scientists who remain at the Institute of Marine Biological Research in Sevastopol celebrated annexation, but many others left Crimea.



in Simferopol, all eight researchers in the Stone Age division, including the institute's former director, left for Kyiv. "We won't be excavating sites from that period now," says Valentina Mordvintseva, a staff scientist. She herself had German funding to excavate a site on Crimea's Kerch Peninsula—until the E.U. sanctions. "I lost the expedition," she says. "It was disastrous."

Valerij Eremeev, the former director of the Institute of Biology of the Southern Seas—IMBR's name before annexation—is now in Kyiv, spearheading an effort to reconstitute Ukraine's marine science after the loss of the two Sevastopol institutes.

The Ukrainian government evacuated to Odessa an elite research team from the University of Nuclear Power Stations in Sevastopol, says Maksym Strikha, Ukraine's deputy science minister in Kyiv. All told, he says, as many as 700 scholars "are working in mainland Ukraine now."

A handful of scientists fled the other way. Igor Dovgal had spent 35 years at the Institute of Zoology in Kyiv, and had risen to deputy director. A protist specialist, he moved to IMBR at the end of 2014. "It was a difficult decision. Even now I'm surprised at myself that I took it," he says. His wife and daughter stayed in Kyiv. But he says he could not stand the anti-Russian feeling in the Ukrainian capital during the 2013–14 protests. "It made my presence in Kyiv impossible."

Russia is making a concerted effort to reward loyal Crimeans. In April 2014, RAS President Vladimir Fortov in Moscow visited Crimea in support, he says, of the "unification" of its top institutes with the Russian academy. IMBR says funds for marine expeditions increased threefold last year, and RAS is showering it with new instruments. Longtime collaborations between Moscow's Institute of Oceanology and MHI diminished when MHI became more involved in European programs. Now, "our doors are

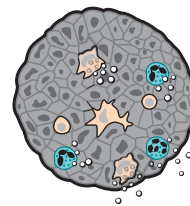
open" to colleagues in Crimea, says Mikhail Flint of the Institute of Oceanology. "We are trying to do our best to help them." Some years ago, MHI had to scrap its four Soviet-era research ships, and had been tagging along on IMBR's ship. "Moscow has promised us a new ship," says MHI's Vladimir Belokopytov.

Although official contacts are barred, scientists in Crimea say they share data with colleagues in Ukraine and Europe, and continue to write joint papers. To Ukraine's consternation, a few papers in Western journals—including ones published by Elsevier and Taylor & Francis—recently appeared listing affiliations as Sevastopol, Russia.

Many scientists here believe that Crimea's pariah status will fade. "We expect the geopolitical situation will change and full scientific relations will resume," says IMBR radiochemist Victor Egorov. Even with Ukraine, he says: "We are one and the same tribe." Kubryakov, a mathematical modeler, is not so sure. He is proud that of MHI's 416 staff members, only one researcher, a native of western Ukraine, departed after March 2014. It may be a long time before Kubryakov sees Ukrainian colleagues as family once again—and before his institute is welcomed back into the European fold. ■



MAP: A. CUADRA/SCIENCE



On a roll. Droplets roll off a superhydrophobic surface.



PERSPECTIVES

SURFACE WEAR

Moving superhydrophobic surfaces toward real-world applications

Standardized wear and durability testing is needed to advance the best materials

By **Xuelin Tian, Tuukka Verho, Robin H. A. Ras**

Superhydrophobic surfaces have received rapidly increasing research interest since the late 1990s because of their tremendous application potential in areas such as self-cleaning and anti-icing surfaces, drag reduc-

tion, and enhanced heat transfer (1–3). A surface is considered superhydrophobic if a water droplet beads up (with contact angles $>150^\circ$), and moreover, if the droplet can slide away from the surface readily (i.e., it has small contact angle hysteresis). Two essential features are generally required for superhydrophobicity: a micro- or nanostructured surface texture and a

nonpolar surface chemistry, to help trap a thin air layer that reduces attractive interactions between the solid surface and the liquid (4, 5). However, such surface textures are highly susceptible to mechanical wear, and abrasion may also alter surface

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chemistry. Both processes can lead to loss of liquid repellency, which makes mechanical durability a central concern for practical applications (6, 7). Identifying the most promising avenues to mechanically robust superhydrophobic materials calls for standardized characterization methods.

A variety of methods have been used to test the durability of superhydrophobic surfaces, including linear abrasion, circular abrasion, tape peeling, blade scratching, sand abrasion, ball-on-disk sliding, oscillating steel ball, and water jet tests (6–9). Although many groups report superhydrophobic surfaces resistant to a certain test, the lack of standardization usually makes comparison of different reported results impossible. An additional issue is that surface wetting is often not characterized in the most useful manner.

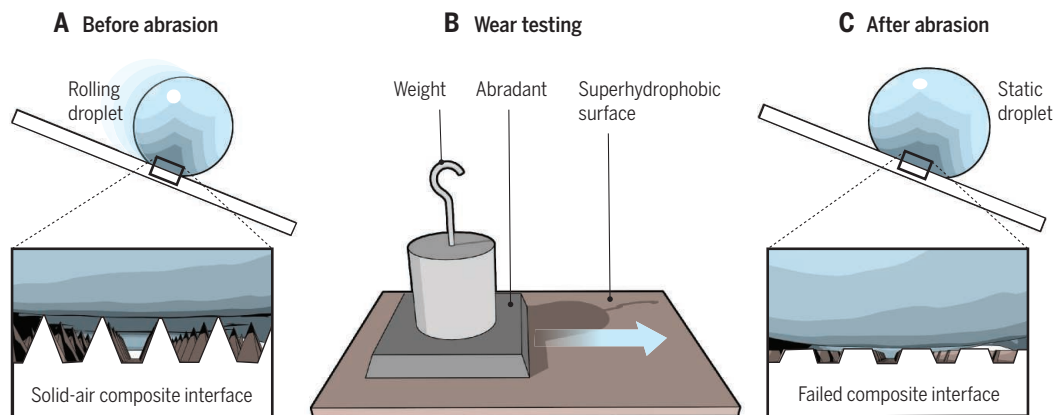
For standardization purposes, a wear-test method should be accessible to most research groups, relevant to most applications, reproducible (that is, insensitive to uncontrolled parameters), and produce a uniformly abraded surface large enough for wetting characterization. The linear abrasion test appears to best fulfill these requirements (see the figure). It involves rubbing a flat solid abrasant against the sample surface under a normal load (7, 10, 11).

Although linear abrasion is already often used for testing the mechanical durability of superhydrophobic surfaces, many studies do not specify sufficient details to facilitate comparison among different materials. The applied normal pressure obviously needs to be controlled. Also, a key parameter is the abrasion distance experienced by each point on the abraded surface, which is the product of the number of abrasion strokes and either the stroke length or the length of the abrasant head (whichever is smaller). A problem may arise if the abrasant head is circular instead of rectangular, as the abrasion distance may then not be uniform over the abraded area. Whether the abrasant moves and the sample stays stationary, or vice versa, is a matter of choice. The effect of abrasion speed may need investigation, but is not expected to be critical.

A difficult matter is the choice of abrasant. In applications, a superhydrophobic surface may be exposed to rubbing of materials with varying hardness, texture, and resilience. Milonitis *et al.* suggested testing a large combination of properties with a set of three materials—textile, rubber, and vitrified (sandpaper) abrasants (7). Such a

test series is reproducible between research groups only when the precise type of each abrasant is well defined; for this, commercial standardized abrasants might present a solution. However, hard abrasants usually cause the strongest wear action, so a simple but satisfactory option that would

materials, so standardized wear testing is highly desirable to accelerate their transfer to real applications. We suggest that linear abrasion should be a primary test and that pressure, abrasion distance, and abrasant materials should be clearly specified. The wear-induced change in contact



Wearing out a nonwetting surface. A superhydrophobic surface generally loses its liquid repellency after mechanical abrasion.

(A) A water droplet rolls on a superhydrophobic surface, where the liquid is suspended by a solid-air composite interface. (B) A setup for linear abrasion test. (C) A droplet gets stuck on the same surface after abrasion because of the failure of the composite interface.

enable community-wide comparison could be the use of silicon carbide sandpapers. Such sandpapers are available with grit size ranging from coarse to ultrafine, allowing determination of the wear response to hard textures with either roughening or smoothing effect.

Even a well-conducted wear test is of little value without characterization in terms of droplet mobility and the advancing and receding contact angles (contact angles to initiate the advancing and receding of a solid-liquid contact line, respectively). Reporting only static contact angles (contact angle after droplet deposition) is common but unfortunately of little value. The static contact angle is not easily affected by abrasion because the advancing contact angle stays high. However, the receding contact angle of abraded surfaces is often quickly reduced, which leads to large hysteresis (difference between the two contact angles) and low droplet mobility (4). It is imperative to characterize the effect of wear in terms of change in contact angle hysteresis or just in the receding contact angle. Alternatively, the sliding or roll-off angle can be used (critical surface inclination at which a sessile droplet starts to move), as it is related to contact angle hysteresis (12). In this case, the droplet volume affects the sliding angle (5) and needs to be reported. Prior to wetting characterization, the surface should be cleaned of debris.

Numerous opportunities are emerging from the study of superhydrophobic

angle hysteresis, receding contact angle, and/or sliding angle should be given. The wear intensity should be incremented up to the point of failure, instead of performing a cursory test and declaring the surface wear-resistant. Although the linear abrasion test is recommended for all superhydrophobic surfaces, additional tests are encouraged—for example, a substrate adhesion test for superhydrophobic coatings (13), a laundering test for superhydrophobic textiles (14), and a water jet test for outdoor (rain) applications. ■

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RIBOSWITCHES

(Meta-)genome mining for new ribo-regulators

RNA regulatory elements are potential antibiotic targets and synthetic biology building blocks

By Morten O. A. Sommer¹
and Beatrix Suess²

Riboswitches are small structural elements within messenger RNA (mRNA) that can change their conformation in response to specific environmental exposures. These changes can alter mRNA transcription or translation. Such ribo-regulators are emerging as a substantial contributor to bacterial gene control. Yet such RNA-based regulation remains challenging to study, in part because of a lack of effective high-throughput technologies for their unbiased identification. On page 187 of this issue, Dar *et al.* describe a novel method for genome-wide experimental identification of genes that are regulated by conditional transcription termination, which likely is a result of RNA structural switching (1). This work further enhances our understanding of bacterial gene regulation and expands the universe of RNA-based regulatory devices that can be deployed in synthetic biology applications.

In 2002, discoveries involving small RNAs were honored by *Science* as the Breakthrough of the Year: “RNA, long upstaged by its more glamorous sibling, DNA, is turning out to have star qualities of its own...” (2). Among the plethora of regulatory RNAs discovered in the past decade, riboswitches (and attenuators) represent a particularly intriguing group of cis-acting regulators. They reside in the untranslated region of the mRNA they control and can fold into mutually exclusive structures. In this way, one structural configuration allows gene expression, whereas the other structural configuration does not. The trigger for this conformational change is often a direct binding of a ligand.

The majority of the riboswitches discovered until the work of Dar *et al.* were found as conserved elements in the untranslated regions of gene transcripts. About 25 different classes of riboswitches have been discovered using this approach and have been experimentally verified in bacteria,

archaea, algae, fungi, and plants (3).

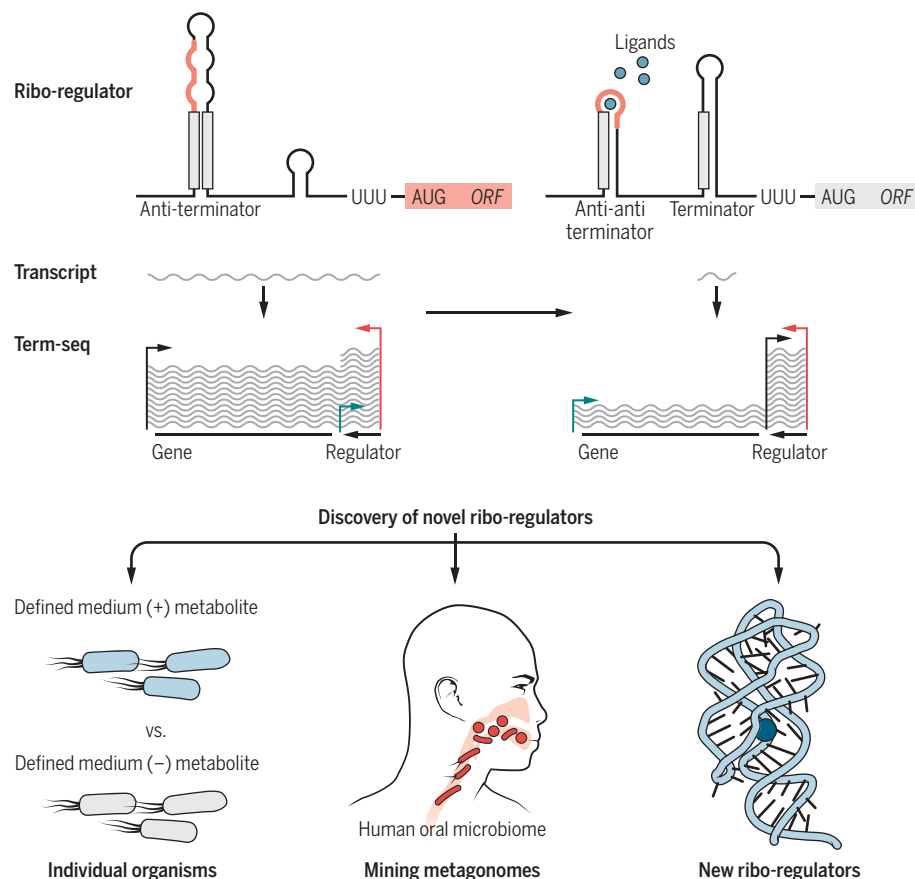
Riboswitches control a multitude of biological pathways, including bacterial vitamin and amino acid biosynthesis. Their mode of gene regulation primarily involves the control of transcription termination or the initiation of translation. However, riboswitches also exist that control splicing, mRNA degradation, or ribozyme activation. Riboswitches selectively respond to metabolites but can also be triggered by temperature or metal ion binding (4).

It is generally believed that the currently known riboswitches represent only the tip

of the iceberg; however, the identification of new riboswitches by conservation-based approaches is not likely to continue to be a driver for discovery. For instance, non-conserved ribo-regulators, which are only present in one species or a specific clade of bacteria, are not likely to be discovered using comparative bioinformatics.

Dar *et al.* present a method that allows the discovery of RNA regulation irrespective of its evolutionary conservation. The methodology combines RNA sequencing with a library preparation method, term-seq, that identifies the 3' end of the transcript. By applying this method to an organism grown in the presence and absence of a particular ligand, genes that are regulated at the RNA level leading to conditional premature termination of transcription can be identified (see the figure). The power of the approach is demonstrated by the identification of more than 90% (49 of 53) of known riboswitches as well as the discovery of 18 new potential regulatory elements in *Bacillus subtilis*.

The majority of antibiotics that target mRNA translation have RNA binding prop-



A ribo-regulator “Big Bang.” Ribo-regulators can regulate the transcription of a gene in response to ligand binding. This regulation leads to a conditional termination of transcription of the gene, such that only the first part of the transcript is completed. Accordingly, a decrease in the full gene transcript will be noted by the term-seq approach of Dar *et al.*, along with a new transcription stop site. In this way, ribo-regulators can be discovered in individual organisms as well as metagenomes, leading to the experimental discovery of ribo-regulators that can be used as new components in synthetic biology.

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erties. Accordingly, it has been suggested that RNA-based regulation of antibiotic resistance genes would be important. The authors identify a wide range of genes that are conditionally terminated by various antibiotics targeting translation. From the perspective of the bacterium, it is important to rapidly activate the expression of an antibiotic resistance gene in response to that particular antibiotic. Having such regulation occur at the RNA level enables exactly this kind of rapid response to antibiotic exposure.

The discovery of such new regulatory mechanisms controlling antibiotic resistance genes provides an improved understanding of antibiotic resistance and also suggests that these ribo-regulators might be potential drug targets. Furthermore, integration of such regulatory elements into reporter systems could create biosensors that enable characterization of drug exposures at the single-cell level. These tools are likely to aid in the study of the mode of action of antibiotics as well as the evolution of antibiotic resistance.

Because the approach developed by Dar *et al.* is dependent only on access to RNA samples, the approach can be deployed to study metagenomic RNA samples. As a proof of concept, the authors sampled the human oral microbiome and exposed samples to various antibiotics. In this way, the authors identified several genes ribo-regulated by antibiotics, highlighting the generality of the approach.

In addition to improving our understanding of bacterial gene regulation and response to antibiotic treatment, the study also opens new avenues for building synthetic biology tools. The versatility and modularity of RNA-based regulation has spurred the development of RNA regulatory devices for building genetic circuits and even creating regulatory elements *de novo* (5). These efforts have led to the construction of complex genetic circuits, yet their applicability remains limited by several factors, including the spectrum of ligands for which RNA regulators exist. Given the immense diversity of the biological world, it is very likely that we have only scratched the surface of this diversity in terms of mining biological systems for regulatory devices that can be deployed in synthetic biology. Approaches such as the one described by Dar *et al.* will allow researchers to expand the repertoire of such elements available to synthetic biologists. ■

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CANCER

How neutrophils promote metastasis

Neutrophils may be cellular targets for cancer therapy

By Thomas Tüting¹ and Karin E. de Visser²

Metastatic disease is the main cause of death in cancer patients. Despite its devastating effects, the complex processes that lead to metastasis are poorly understood. During their journey to distant organs, cancer cells encounter various types of normal cells, including immune cells. Neutrophils are the most abundant immune cells in our blood, and they protect us from infections and facilitate wound healing. Intriguingly, neutrophils frequently accumulate in cancer patients. Recent studies have addressed the causal link

“Are tumor-associated neutrophils just innocent bystanders...or do they actually influence cancer behavior?”

between neutrophils and cancer in mouse tumor models, and point to a key role of neutrophils in promoting the most deadly aspect of cancer—its dissemination to distant organs (1–4).

Observations in cancer patients have linked elevated neutrophil counts in blood with increased risk for metastasis (5). Furthermore, ulceration of melanomas and subsequent neutrophilic inflammation are associated with invasiveness and a high probability for metastatic dissemination (1). Notably, a gene expression meta-analysis of ~18,000 human tumors across 39 cancer types linked an intratumoral neutrophil-related gene signature with poor prognosis (6). Are tumor-associated neutrophils just innocent bystanders that accumulate in aggressive tumors, or do they actually influence cancer behavior?

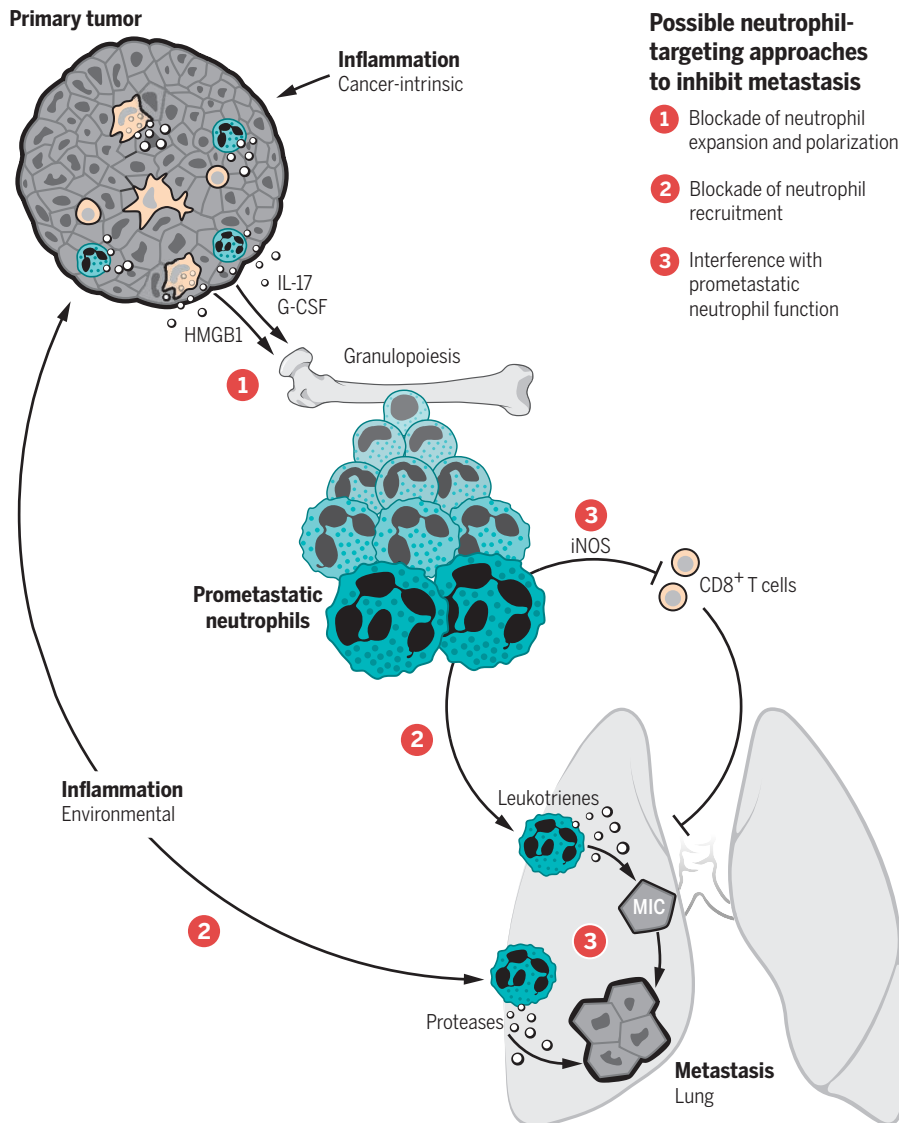
In two independent transgenic mouse models that mimic human breast cancer,

primary breast tumors induce neutrophil accumulation in distant organs before the arrival of cancer cells, where they enhance the early steps of metastasis formation (2, 4). In one model, neutrophils localize to the lung where they produce leukotrienes that facilitate colonization by selectively propagating cancer cells with higher tumorigenic potential (4). In the other breast cancer mouse model, neutrophils promote lung metastasis by dampening antitumor T cell immunity (2). In both studies, neutrophil accumulation in the (pre)metastatic niche was initiated by signals emanating from the primary tumor.

Environmental stimuli can also trigger the prometastatic functions of neutrophils (1, 3). In a transgenic mouse model of melanoma, ultraviolet irradiation induced neutrophil activation in the skin, which promoted invasive and migratory behavior of melanoma cells, resulting in their expansion along blood vessel endothelial surfaces and distant metastasis formation (1). Additionally, bacterial lipopolysaccharide-induced acute lung inflammation initiated recruitment of neutrophils, which release proteases that can degrade thrombospondin-1, a matrix glycoprotein. Thrombospondin-1 inhibits tumorigenesis; thus, its destruction enhances metastatic outgrowth (3). Taken together, neutrophils can exert prometastatic functions in response to an inflammatory trigger from either the primary tumor or an environmental stimulus.

These experimental and clinical findings provide a scientific basis for therapeutically targeting the prometastatic role of neutrophils in cancer (see the figure). Importantly, such approaches must be developed with caution because neutrophils also exert anti-metastatic activity in other experimental mouse models (7, 8). The ability to inhibit or promote tumor growth and metastatic spread of cancer cells in different experimental systems illustrates the context dependency and plasticity of the neutrophil phenotype. It is generally believed that progressively growing tumors perturb the process of granulopoiesis in the bone marrow, and switch neutrophils from tumor-protective to disease-promoting, more-immature phenotypes (9). The molecular and cellular mechanisms orchestrating this

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Prometastatic neutrophils. Molecules emerging from primary tumors or inflamed tissues can promote the emergence of protumorigenic neutrophils from the bone marrow. Neutrophils can facilitate metastasis through multiple mechanisms, such as the release of proteases that degrade antitumor factors, and leukotrienes that propagate metastasis-initiating cells (MIC). Protumorigenic neutrophils also can stimulate cancer cell expansion along blood vessel endothelial surfaces (not shown) and suppress antitumor T cell responses. Several neutrophil-targeting approaches could potentially inhibit metastasis, as shown. iNOS, inducible nitric oxide synthase.

phenomenon are only partially understood and represent a key challenge for current research. So far, it has become clear that molecules such as high mobility group box 1 (HMGB1) (1), interleukin-17 (IL-17) (2), and granulocyte colony-stimulating factor (G-CSF) (2, 10) promote the emergence of protumorigenic neutrophil phenotypes. Most likely, the genetic makeup and type of tumor are key determinants dictating the amount of these and other neutrophil-educating mediators that are produced.

The selective interference with prometastatic neutrophil functions represents an attractive new strategy for cancer treatment. For example, an inhibitor of the arachido-

nate 5-lipoxygenase (the enzyme that transforms essential fatty acids into leukotrienes, which promote inflammation) impairs the formation of lung metastases (4). Lung metastases also can be inhibited by targeting neutrophil proteases (3). Interference with systemic inflammatory mediators that promote the emergence of prometastatic neutrophil phenotypes, such as IL-17 and G-CSF, represent an alternative treatment approach (2). Along the same lines, pharmacological blockade of endogenous innate immune-activating molecules such as HMGB1 or downstream signaling pathways such as Toll-like receptor 4 (TLR4) also abrogate neutrophil mobilization (1).

Importantly, the oncology field does not need to develop neutrophil-targeting agents, as it can benefit from compounds that have already been developed for treating inflammatory diseases, such as the asthma inhibitor zileuton (4). In addition, an IL-17 inhibitor has recently been approved for the treatment of psoriasis. Clearly, more experimental work is needed to better understand which features of tumors and which types of inflammatory environmental stimuli can induce prometastatic neutrophils and during which step of the metastatic cascade therapeutic intervention will be most effective.

Although preclinical studies suggest that monotherapy with neutrophil-targeting compounds is sufficient to inhibit metastasis (1–4), their use in cancer patients could be more effective when combined with well-established anticancer strategies such as chemo- and radiotherapy or targeted inhibition of oncogenic signal transduction. Based on the frequently observed immunosuppressive phenotype of tumor-educated neutrophils (2, 10), there is great potential in combinations with new cancer immunotherapy approaches that are currently revolutionizing the treatment of patients with various types of advanced cancer. This concept is supported by emerging evidence from clinical studies showing that high numbers of neutrophils relative to lymphocytes in the blood of melanoma patients are associated with poor response to immune checkpoint inhibition (11, 12), and from experimental studies in mice showing that neutrophils impair successful cancer immunotherapy (13, 14). New insights into the diverse mechanisms by which neutrophils promote metastasis will undoubtedly broaden the scientific basis for deploying neutrophil-targeting agents as part of multimodal treatment approaches for patients with advanced cancer in the near future. ■

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How to overcome prejudice

A brief conversation can have a lasting effect on prejudice

By Elizabeth Levy Paluck

What do social scientists know about reducing prejudice in the world? In short, very little. Of the hundreds of studies on prejudice reduction conducted in recent decades, only ~11% test the causal effect of interventions conducted in the real world (1). Far fewer address prejudice among adults or measure the long-term effects of those interventions (see the figure). The results reported by Broockman and Kalla on page 220 of this issue are therefore particularly important (2). The authors show that a 10-min conversation with voters in South Florida reduced prejudice against transgender people and increased support for transgender rights for at least 3 months.

As the authors acknowledge, these strong results in the wake of a brief intervention might seem surprising. But readers may find it even more surprising that so few previous field studies have tested the causal effect of any type of intervention, aimed at any type of prejudice. Experimental tests of interventions to reduce prejudice have usually been confined to the laboratory. Field studies have mostly measured individuals' levels of prejudice with ever more sophisticated surveys.

Broockman and Kalla's results thus do not represent a new challenge to an established field: They stand alone as a rigorous test of this type of prejudice reduction intervention. The authors combine a rigorous field experiment with long-term, high-quality measurement of its outcomes. Their exciting methodological template is now available to other investigators, allowing them to test how canvassing interventions affect prejudices and political attitudes (3).

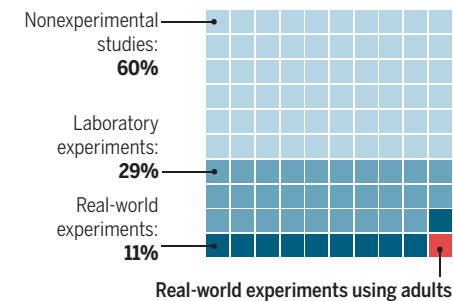
The results of the study align with psychological theories and empirical demonstrations that prejudice is subject to peer influence (4), fluctuations in perceived social or personal threats (5), and the structure of educational group tasks (6). They stand in contrast to those who have argued that individual prejudice is resistant to change (7).

How should we understand the nature of prejudice, particularly its relationship to political attitudes and behaviors? One of the best

ways to approach this question is by studying the successes and failures to change prejudice among various populations in the world. Broockman and Kalla's study represents an important advance for this approach. They randomize whether voters are visited by a canvasser to discuss transgender rights or recycling (control), and further, whether that canvasser is transgender or nontransgender. Their results show that the carefully-scripted discussions led by both transgender and nontransgender canvassers led to the observed changes, even when study participants watch political attack ads.

It remains to be shown whether the scripted discussions were successful because

Prejudice reduction literature



Prejudice reduction. The finding that canvassing can reduce prejudice toward transgender people (2) adds to a small literature testing the causal effects of real-world prejudice reduction interventions. As of 2009 (1), 11% of published and unpublished studies experimentally tested the effect of interventions conducted in the real world.

they asked voters to recall a time when they were judged negatively to understand a transgender person's perspective ("analogic perspective-taking"). Rather than investigating the psychological processes responsible for the effect, Broockman and Kalla focus on whether the canvassing intervention produced substantive and durable changes that are detectable in a nonlaboratory environment. This is a welcome development: Social scientists have spent enough time in the lab learning about the mechanisms of interventions with no known real-world effects (1).

Analogic perspective taking is not the most prominent method in the prejudice reduction literature. Activists at the LA LGBT Center developed the intervention by testing different persuasion techniques over more than 13,000 canvassing conversations (8). The cur-

rent study's success speaks to the promise of a social science that takes the hypotheses of experienced practitioners seriously.

Broockman and Kalla also tested the contact hypothesis, according to which contact with a member of a stigmatized group reduces prejudice toward that group. Psychologists have studied this idea in hundreds of correlational studies and laboratory experiments (9). However, Broockman and Kalla did not find a statistically significant difference between the effect of transgender and nontransgender canvassers. This null finding contradicts the most optimistic predictions of the contact hypothesis. If in fact there is no difference, this is good news for stigmatized groups that are a demographic minority and require outsiders to help campaign on behalf of the group. This is an exciting question to address in future field experiments.

Even when it is driven by a respected theory, an intervention lasting just 10 min may seem too minor to produce substantial effects. Findings of large effects caused by small, theory-based interventions have attracted discussion in recent years (10). However, in the case of Broockman and Kalla's study, we might question whether the intervention is in fact unusually minor. The 10 min consisted of a conversation with a stranger about a memory of personal vulnerability and its relevance to a social issue. A conversation like this seems to be one that people seek out: Individuals report confiding in and discussing important matters with relative strangers, especially if the person is considered knowledgeable on the topic (11).

Furthermore, a face-to-face conversation is not minor when compared with other interventions used to influence political or social attitudes, like 1- to 2-min mass media advertisements (12). Considering both the absolute and relative importance of such a conversation, it seems plausible that a meaningful interaction could take place in a short period of time. Social scientists would do well to continue collaborating with practitioners on the design and study of these brief but meaningful interactions. ■

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DIVERSITY IN SCIENCE

A recipe for change: Creating a more inclusive academy

Using data, selecting leaders, and changing rules

By **Beth Mitchneck**,¹ **Jessi L. Smith**,²
Melissa Latimer³

Although there has been a welcome increase in discussion about gender disparities in science, technology, engineering, and mathematics (STEM), broad participation of women from all backgrounds in academic STEM will not be achieved until institutions are transformed. A long-range view is needed to change the rules of the game, such that institutional culture and practices create work-

places where all scientists and engineers want to be. We lay out a six-point plan of what needs to change, who should participate, and how actors outside of the academy should have direct involvement in the process.

We focus on gender but recognize the importance of attending to gender identity, ability, race and ethnicity, class, sexual orientation, and other important intersections.

Changes that bring about inclusion for one group, we argue, can have far-reaching benefits for everyone.

Learn the social science research. The entire campus community must be better informed about hurdles to hiring, retaining, and promoting women, especially women of color. Decades of social science research

show processes through which explicit and subtle bias operate (1, 2) including within academic science (3, 4). Subtle bias is especially problematic, as it operates without awareness, particularly when people are cognitively taxed and busy (2). Biases must be disrupted in order to prevent the status quo from inevitably reproducing itself. Yet even with the best intentions, the bias “habit” is hard—but not impossible (5, 6)—to break. Breaking the habit at one level does not necessarily mitigate bias at another level of evaluation or experience (7).

You cannot stop at just documenting bias, as those data can be met with suspicion (8); arm yourself with an understanding of the myriad ways in which bias contributes to stereotype threat, belonging uncertainty, work-life imbalance, and a host of other negative outcomes (9, 10). Transformation cannot begin until people understand that bias in all shapes and sizes exists within faculty, leaders, and the structures therein (for a list of resources about bias in all its guises, see: wiseli.engr.wisc.edu/library.php).

Leaders must understand the context and be accountable for diversity and inclusion. It is not a good idea to try to change an institution with which you are not highly familiar. Outside consultants and new leaders have a place, but the unique history and sociopolitical dynamics of an academic institution must be considered when selecting and enacting change strategies (11). We need to select leaders from all levels of the institution who understand the context, are accountable for implementing change, and make it their mission to promote diversity and inclusion. Leaders must be ready for and able to withstand pushback. A president or provost does not have to lead the transformation but does need to be a visible, vocal part of the change process and set up an accountability system (e.g., including successful diversity outcomes as part of how their effectiveness as leaders is evaluated).

Every type of leader should be represented in change efforts. Inclusion of faculty members and thought leaders from all genders and backgrounds makes for a more effective process, because people are more likely to process information with an open mind if the communicator is someone with whom they typically agree or identify (12). It is essential to have leaders who communicate in ways that faculty can hear.

Seek external catalyzing resources. Funding agencies and private foundations that partner with universities add legitimacy to institutional transformation. The U.S. National Science Foundation (NSF) has committed well over \$130 million to increase the participation and advancement of women in academic science and engineering careers

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through the ADVANCE program alone toward funding gender-equity change initiatives. Private foundations, such as Sloan and Elsevier, provided partnerships and incentives to leaders undertaking institutional transformation. The European Commission takes institutional transformation around gender equity so seriously that its Horizon 2020 program has an expert group on gender to help promote research and innovation. Universities, however, must feel an intrinsic desire to transform, and many changes should be made even without funding. Ultimately, universities must make their own investments to ensure that activities lead to sustained institutional change.

Focus at the department level. Many barriers to cultural change are embedded in institutional practices [e.g., recruiting and promotion and tenure (P&T)] and manifested at the academic-unit level. Thus, efforts to address departmental climate and social interactions are critical (13). A helpful, fair, inclusive department where faculty speak daily with colleagues about research prospects and interests increases their likelihood of having clear perceptions of P&T

“Changes that bring about inclusion for one group...can have far-reaching benefits for everyone.”

evaluation (7). Departmental interventions to promote inclusive decision-making and effective communication improve key aspects of climate critical to women's success (13). Intervening with department-level faculty search committees in ways that meet their basic psychological needs of competence, relatedness, and autonomy resulted in changes to search processes and outcomes (6). There are many other inputs into department-level climate to target, including teaching and committee assignments, conflict management, mentoring and networking activities, and so on (see www.colorado.edu/eer/research/strategic.html).

Collect and publicly share data. As institutional changes occur, development of a new narrative about the success of institutional change engages people to attempt more change. Construct and maintain “change narratives” as a way to initiate and sustain the change momentum and focus on gender equity (14).

Public discussions of where an institution is, where it wants to be, and how to get there means taking a hard look at data. Systematically collecting and actively sharing data about the change process creates necessary

visibility for assessing short- and long-term impacts of initiatives (15) that can bring the university community on board. Beyond the Integrated Postsecondary Education Data System (IPEDS) data sets available through the U.S. National Center for Education Statistics, many NSF ADVANCE institutions have developed publicly available data sets on faculty diversity that are lacking at other universities. Dedicated institutional research staff can be accountable for collecting, analyzing, exhibiting, and engaging stakeholders with equity data.

When funders require data as an award condition, universities usually comply. Funding agencies could require data on gender and racial equity as part of proposal submissions. Some professional associations work successfully with the university community to collect data on diversity, like the Computing Research Association and the Collaborative on Academic Careers in Higher Education (COACHE) at Harvard. Partnerships help to promote visibility and institutional change. What it takes is devoting resources—time, effort, and accountability—to oversee data collection and public sharing.

Policy change is critical. A move toward all-inclusive multiculturalism requires using inclusive language in all communication and work policies (16) and is associated with positive changes in the work climate. One area in which policy change has occurred is work-life support. For example, changing the stop-the-tenure-clock policy to be “opt-out” instead of “opt-in” increases use of the tenure extension and reduces the burden and stigma of having to ask for an accommodation (10).

Work-life policies are essential in recruiting, retaining, and advancing high-quality faculty (10) so long as there is not a “flexibility stigma” in which people are subtly or blatantly penalized for using work-life friendly policies (16). Such policies provide flexibility to balance work and life obligations and to ensure career progress for all faculty, regardless of sex, marital status, sexual orientation, race and/or ethnicity, or academic discipline (10). After showing success with simpler policy changes—such as work-life support—change agents must turn to policies that target a system filled with gender-biased and racialized barriers to inclusion.

Status quo P&T policies might be the single greatest hurdle to change. Although the professoriate has changed dramatically over the past 20 years, many embedded values and expectations for what counts and the timeline for when things count toward advancement have remained fairly static. P&T policies are often inflexible and largely reduced to an exercise of counting publications, external funding, and impact factors. This reductionist evaluation can hurt women faculty, who

are often drawn to collaborative teams and interdisciplinary research (17). Although such team-based science is increasingly necessary for innovative success, the P&T process is more likely to reward sole authorships published in mainstream journals funded by primary investigator-led grants. Women's contributions to team science are disproportionately discounted (18), and team science projects may take longer and be published in less traditional outlets. What is good for science is for faculty and professional societies to work together to consider how P&T policies could be changed to acknowledge and reward various pathways to success.

Innovation and global competitiveness are compromised by excluding women and minorities from the professoriate (19). True transformational change means changing what it means to be an academic and who belongs in the academy. The barriers and those cultures, practices, and structures that require transformation are well researched as are remedies. Now it's time to act.

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BIOMOLECULAR FOLDING

Moments of excitement

Direct measurements of protein folding paths agree with theoretical predictions

By Peter Wolynes

At the atomic level, biomolecular dynamics, like war to its soldiers, consists of long periods of boredom interspersed by brief, intense moments of excitement. During the vast majority of its existence, a biomolecule flails about randomly, with large-amplitude motions when unfolded and with smaller-amplitude motions when folded. Rare transitions between these two states of often apparently aimless activity occur through a fleeting series of steps: a set of transition paths guided by the biomolecular energy landscape. On page 239 of this issue, Neupane *et al.* use single-molecule force spectroscopy to study the transition paths for folding a nucleic acid and for misfolding a prion protein (1). They are able to confirm some very basic aspects of biomolecular energy landscape theory.

Much of our knowledge of how molecules fold has come from painstaking measurements of the typical length of time that biomolecules spend in their states of boredom under many thermodynamic conditions,

and then using our imagination (disciplined by theory and computation) to fill in the gap of how the molecule actually moves between its folded and unfolded ensembles (2). Recently, however, dynamical fluorescence spectroscopy has made the exciting moments of transition between folded and unfolded states directly accessible to experimental observation (3). These studies have provided estimates for the typical time involved in a folding transition.

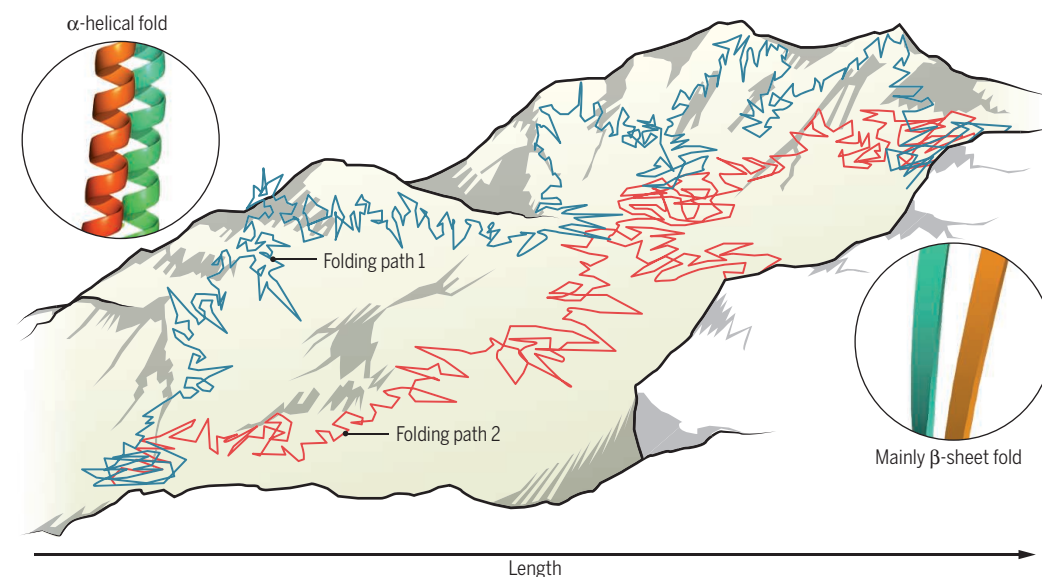
Neupane *et al.* use a different technique, single-molecule force spectroscopy, in which a force is exerted on a single biomolecule while simultaneously monitoring its length with subnanometer precision as it repeatedly unfolds and refolds. The extraordinary stability of the apparatus allows tens of thousands of transitions to be monitored over a period of hours. Each folding or unfolding transition takes place in only a few tens of microseconds; the molecule spends vastly more time simply ambling about. By monitoring the length during these transitions as a function of time and then analyzing the specific time histories, the authors build up a one-dimensional view of how each molecule folds. Even glancing at a few such histories settles a long-standing question: Is there a unique folding pathway?

The authors find that not all histories are the same, and there is thus no evidence for an obligate single folding pathway. Instead, the authors find a multiplicity of paths, as has come to be expected from modern energy landscape theory.

A more quantitative test of energy landscape theory is provided by measuring the statistics of the transitions. Biomolecular folding involves random diffusion on an energy landscape with many dimensions. Nevertheless, this motion can often be projected onto a single coordinate such as the length, especially when the energy landscape is funneled. For funneled landscapes, the fraction of native structures acts as a good reaction coordinate, monitoring the reaction's progress. After projection, the motion is no longer completely random, but rather is guided by free energy gradients. The diffusion coefficient for this motion measures how easily and how often the molecule can escape from local minima on the full many-dimensional energy landscape.

The projected free energy surface typically has a barrier, because the entropic cost of organizing the molecule is not immediately repaid by increased stability of conformations with partially formed correct native structure. The free energy barrier explains why the waiting times are much longer than the time to traverse the barrier. By pulling continuously and measuring the work needed to unfold the molecule, the thermodynamic free energy profile can be measured. According to theory, the transition time scales inversely with diffusion rate, and measuring the typical transit time therefore gives a good estimate for diffusion rates. The overall waiting time, however, not only depends on diffusion but also scales exponentially with the thermodynamic barrier, which makes the waiting time so long.

Neupane *et al.* find that their single-molecule measurements of the barrier, waiting times, and transition times agree in the main with the simplest one-dimensional theory. The kinetic barrier for folding, however, turns out to be smaller than the thermodynamically determined one. The authors trace this discrepancy to an excess of short transit times relative to the expected simple exponential form for a strictly one-dimensional problem (4).



More than one path. This schematic two-dimensional free energy plot shows that the free energy of a protein under force depends on the fraction of β sheet-like secondary structure as well as on the molecule's length. The latter is the only coordinate monitored by Neupane *et al.* The protein can follow different paths during folding; two possible paths are superimposed on the plot.

This apparent excess could come from unwanted influence of the Brownian motion of the tethers that pull on the molecule, or it might indicate that more coordinates of the biomolecule in addition to the length are needed to describe the transition. Such an explanation would not be surprising, because the length coordinate is not expected to be perfectly correlated with the folding or misfolding reaction coordinate (see the figure). The protein system studied by Neupane *et al.*—a prion—also probably does not have a well-funneled energy landscape, given that its configurational diffusion is extremely slow. For rugged landscapes, theory suggests that the logarithms of the escape times nearly follow a normal Gaussian distribution. This distribution gives a wider range of transit times than the prediction for strictly one-dimensional diffusion (5).

Now that Neupane *et al.* have directly confirmed some of the most basic notions of energy landscape theory by observing transition paths, we can expect future refinements to give more structural details

“...biomolecular folding is now on its way to becoming one of the best-understood processes in biochemistry.”

about the transitions. To access these details, one can simultaneously measure fluorescence and length (6). However, existing measurements of this sort must be extended in time range and stability to uncover the multidimensional aspects of folding transition paths. Even without such enhancements, combining the capabilities of protein engineering with transition path measurement will give direct access to the structural aspects of the transition path ensemble. These structural factors have been predicted by theory and simulation for many proteins (2). Leaving its days of controversy, biomolecular folding is now on its way to becoming one of the best-understood processes in biochemistry. ■

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CLIMATE CHANGE

A heated mirror for future climate

Climatic changes 55.9 million years ago resemble those expected in the future

By Richard B. Alley

Climate has always changed naturally, and this is not good news when contemplating a human-forced future. The natural responses have been as large as, or larger than, those simulated by leading models for shorter time scales, with major biological and physical impacts. The possible effects of rapid carbon dioxide (CO₂) release may be clearest from the Paleocene-Eocene Thermal Maximum (PETM) about 55.9 million years ago, when a large, natural CO₂ release drove strong warming that caused amplifying feedbacks, dwarfing of large animals, ecosystem disruptions, soil degradation, water-cycle shifts, and other major changes (see the figure). The climatic changes during the PETM occurred over longer time scales than those of anthropogenic climate change. The impacts of the latter may thus be even more severe.

The source of the initial CO₂ release that drove the PETM remains debated (1). Increasing evidence points to a concentrated igneous outpouring during the opening of the North Atlantic, which intruded oil-bearing and otherwise carbon-rich rocks (2). The PETM was amplified and extended by sustained CO₂ release, probably at least in part because the warming released organic carbon stored in soils, seafloor sediments, or elsewhere (1).

Most estimates of the total CO₂ added to the atmosphere during the PETM are similar to, or somewhat lower than, the total CO₂ that would arise from burning all fossil-fuel resources estimated to exist on Earth—especially if, as suggested by the PETM and by current understanding, warming releases additional carbon from reservoirs such as tundra soils and seafloor hydrates (1). However, the initial CO₂ rise during the PETM took place over the course of a few millennia, about a factor of 10 slower than if humans burned the remaining fossil-fuel resources under a business-as-usual scenario (3). PETM CO₂ remained elevated for more

than 150,000 years, confirming the long persistence expected for human-released CO₂ (1).

The strong PETM warming suggests that climate is highly sensitive to rising CO₂. This implies a higher climate sensitivity than the lower end adopted for somewhat shorter times by the Intergovernmental Panel on Climate Change (IPCC), and perhaps larger than the higher end (4). Thus, temperatures may rise more than currently projected.

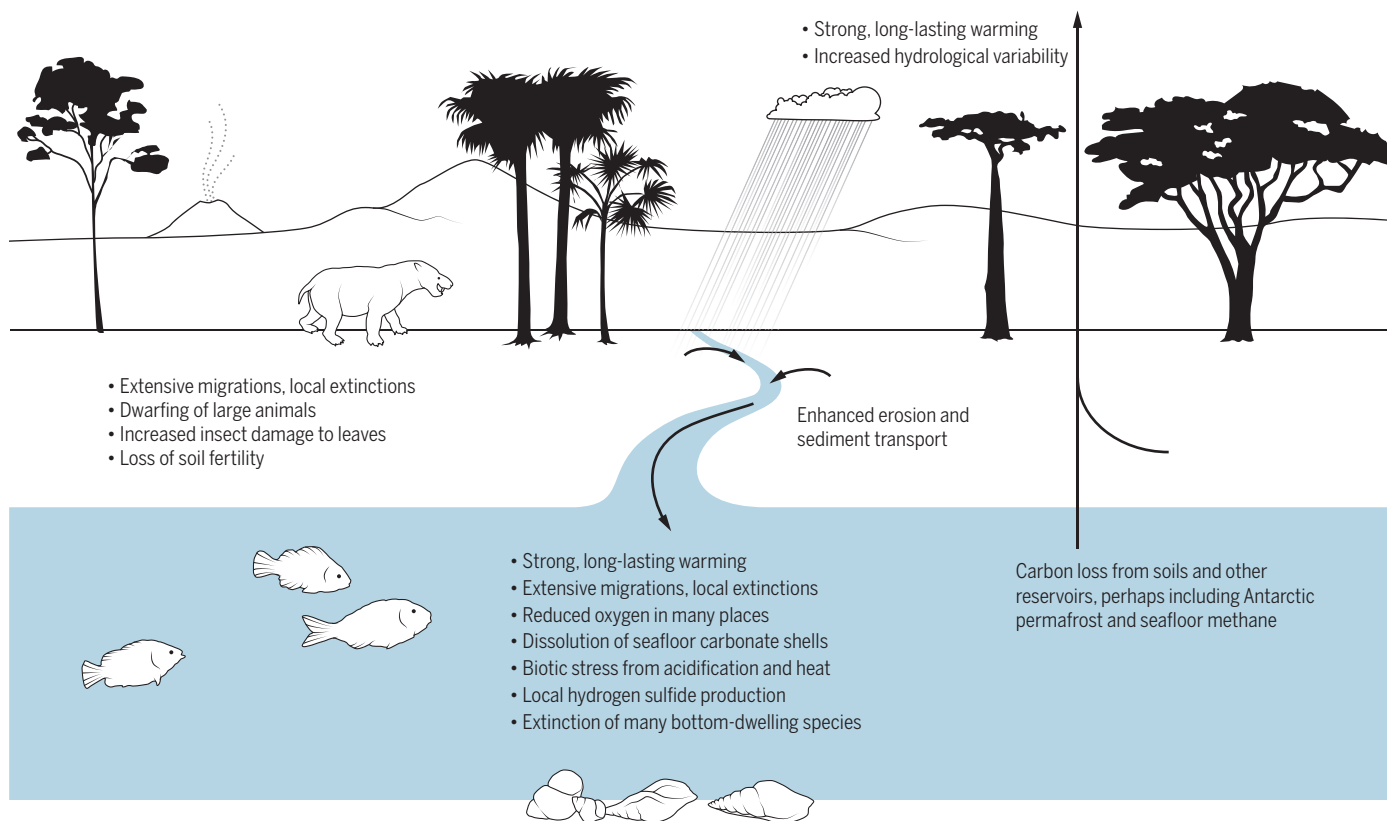
During the PETM, the rise in CO₂ and resulting climate shifts caused further changes propagating across the Earth system. On land, enhanced erosion and sediment transport to the sea (5, 6) are consistent with the expected increase in hydrological variability from warming; larger or more intense storms separated by longer and drier intervals likely contributed to regional loss of vegetation, soil carbon, and soil fertility (5).

Over the course of the PETM, terrestrial species migrated long distances poleward or upward and crossed land bridges between continents. Some types became extinct, whereas others spread. Ecosystems during the event were notably different from those before or afterward. Wing and Currano have shown that of a sample of 91 common plant taxa known from fossils during a million-year-long interval starting 200,000 years before the PETM in Wyoming's Bighorn Basin, only 7 persisted before, during, and after the PETM. Another 12 experienced at least local extinction at the onset, 20 were confined to the event, 40 were locally absent during the event but present before and after, and 12 first appeared after the event (5).

PETM plant leaf fossils from the Bighorn Basin are almost twice as likely to show insect damage as the average from before and after; one PETM leaf shows 10 different types of damage. Possible reasons include increased insect feeding as higher CO₂ reduced nutritional value of plants, invasion by new insects, and disruption of established ecological balances (7). Heat and water stress and loss of soil fertility likely also challenged plants (5). Large mammals became notably dwarfed, perhaps because of heat stress or the lower nutritional value of their food (8).

In the ocean, the high CO₂ levels during the PETM raised acidity while ocean warming

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Clues to the future? About 55.9 million years ago, a rapid rise in greenhouse gas concentrations in the atmosphere had major impacts across the planet. Today, greenhouse gas concentrations are rising even faster than they did then.

increased stratification. Low-oxygen zones expanded and anoxic, H_2S -producing conditions developed at least seasonally in some places (9). Up to half of the bottom-dwelling foraminifera species became extinct, and some other types were lost elsewhere, with widespread migrations (1). Coral reefs were largely lost as functioning ecosystems, with the oceans instead hosting flatter, less complicated structures dominated by foraminifera (10). Limited data suggest additional major biological impacts, perhaps including partial or complete loss of many types of plankton in some coastal tropical oceans because of heat or other stresses (6).

Slower climate changes allow more time for biotic adaptation or migration. A slower pace of change also gives time for rising CO_2 to dissolve seafloor shells and break down land-surface rocks, thus supplying calcium and bicarbonate and reducing impacts of acidification on creatures with carbonate shells. Hence, the biological impacts of the PETM were likely less severe than those of human-caused emissions under a business-as-usual scenario.

The PETM stands out in paleoclimatic records, but several other somewhat similar although smaller events over the next few million years provide additional supporting evidence. In these events, features of Earth's orbit, perhaps aided by volcanic forcing, ap-

pear to have pushed the climate system past some threshold, triggering feedback loss of stored carbon that amplified the warming, as in the PETM (11).

The PETM poses major research challenges, but confidence is growing rapidly that studies targeted at key questions can greatly reduce the uncertainties. The sources and rate of rise of the atmospheric CO_2 concentration are still poorly known, but progress in using boron records and other techniques to estimate past CO_2 levels is more tightly constraining the total mass of CO_2 that was released (12). This result, together with knowledge of the timing and size of the shift in carbon isotopic compositions in terrestrial and marine settings, can provide a more accurate history of CO_2 sources (13). Collection of further high-resolution records, including those on land and in shallow marine settings with rapid deposition, could clarify these key issues. Records of ecosystem changes are still available from only a few locations, and many deposits of this age remain to be explored for physical or biological changes.

Narrowing the uncertainties about this important climate event and other similar features in the geologic record could provide additional valuable insights to inform decisions on our energy future. What is clear, however, is that the large release of CO_2 dur-

ing the PETM transformed conditions on land and in the ocean in ways that affected the Earth system for more than 100,000 years and that might be considered catastrophic by many people today. The history of the PETM shows that our decisions will have large and long-lasting consequences. ■

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BOOKS *et al.*

ENTREPRENEURSHIP

Smart start

Practical advice abounds in a faculty-focused guide to commercializing research

By Yael V. Hochberg

The pressure on universities to commercialize the technologies developed in their laboratories has been steadily increasing over the past several decades. The political establishment, having already substantially compressed the funding for basic research, is beginning to apply pressure to show returns on federal research-funding dollars—returns that are often measured using the metrics of jobs created and companies started. Separately, increasing costs associated with university administration have created pressure to generate revenue to cover the often-onerous costs of filing and supporting university-generated patents.

At the same time, faculty have become accustomed to a service model in which patenting of their discoveries is provided without question, and the move to a first-to-file system has further solidified these expectations. The need to file provisional patents before public disclosure, often before a market assessment of the technology can be properly completed, intensifies the pressure. Adding to all of this is the requirement that universities share any returns from the commercialization of resulting intellectual property (IP) with the inventor, which has led many researchers to hope that patenting their academic research will also lead to financial windfall.

Commercializing university technology has long taken multiple tacks, with the bulk of IP-related revenue generated through licensing to large corporations. Not all inventions are appropriate for licensing to established enterprise, however. Commercialization of radical innovations and those whose further commercial development is tied closely to the tacit knowledge of their inventors has traditionally happened through the creation of new startup companies.

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Do you have what it takes to turn a promising idea into a profitable endeavor?

For most academics, the path to the creation of a successful startup is a black box, and often their instincts, honed in an academic system that optimizes for basic research production, can lead them in precisely the wrong directions. It is here that *Research to Revenue* seeks to make its mark.

Rose and Patterson provide university researchers a solid introduction to the process of commercializing laboratory-discovered innovations through the startup approach. With an emphasis on best practices at the various stages of the process, the book presents the harsh realities of startups, including the particular challenges faced in commercializing early-stage technologies that were not necessarily born from a specific market need and the very real (and sometimes uncomfortable) truths about the appropriate role for the faculty member in the process (spoiler: don't plan on being chief executive officer—or even necessarily chief science officer or chief technology officer).

Faculty members often have a very rosy view of the startup process and overvalue their scientific expertise versus the business and market expertise needed to commercialize technologies and build successful, revenue-generating companies. Customers respond to products and services that solve an important problem at reasonable cost in a manner that is superior to existing solutions. Venture capital (VC) investors—who will typically be required for commercializing university technologies—invest in people, business experience, and market pull, not technology per se.

Research to Revenue succeeds in emphasizing the need to consider what important market need the technology can solve, as well as the importance of identifying co-

Research to Revenue A Practical Guide to University Start-Ups

Don Rose and
Cam Patterson

UNC Press, 2016. 352 pp.



founders with appropriate business knowledge and experience to lead the startup through the commercialization process.

Readers will wish to consult other sources for additional information on some elements of the startup process, especially fundraising from outside investors, term sheets, and the interplay between terms and valuations. The topic of angel and VC financing and terms receives extremely limited treatment in this book. Given the substantive popular press on the subject of financing, however, the authors' choice to dedicate the bulk of the discussion to elements of the process that are unique to the university setting is appropriate.

Research to Revenue is likely to be more valuable for faculty members than for university administrators interested in improving their own commercialization activities. Best practices and guidelines for improving technology transfer are not given the same in-depth treatment as the rest of the material covered—an unfortunate omission given that such guidance appears to be sorely needed and sought after in many institutions. That said, academic researchers interested in the university startup process will find *Research to Revenue* an invaluable guide in their quest to take their discoveries from the lab into the world.

10.1126/science.aaf4217

Predicting peak oil

A new biography probes M. King Hubbert's controversial mid-century energy pronouncement

By Charles Hall

We have no choice but to proceed into a future which we may be assured will differ markedly from anything we have experienced thus far. Among the inevitable characteristics of this future will be the progressive exhaustion of the mineral fuels" (1). These words sound very much like what you might hear in various quarters today, but they were written in 1949 by the American geophysicist Marion King Hubbert.

Hubbert is best known for developing the theory of "peak oil": the idea that oil production will be characterized by an initial period of exponential growth, followed by a peak (or peaks), followed by a substantial decline. Debates about Hubbert's predictions were intense in his lifetime and continue today.

I once heard Hubbert introduced at a lecture in this way: "In an age of publish or perish, he has published only nine papers. Each of them is in a different field. And each of them is considered a classic." *The Oracle of Oil* is a masterful telling of Hubbert's life and ideas, written by journalist Mason Inman, a knowledgeable and graceful storyteller.

Hubbert grew up on a hard-scrabble farm in Texas at the turn of the 20th century. At the age of 17, he sold his cow for train fare and set off to Weatherford Community College, where he worked odd jobs to afford the tuition. With the encouragement of, and a loan from, Weatherford's president, he went on to study geology at the University of Chicago. He had intended to study chemistry, but the long laboratory hours conflicted with his job as a post office clerk. After completing his graduate studies, he accepted a position at Columbia University. In New York, he was greatly influenced by the intellectual ferment and Bohemian lifestyle

of Greenwich Village—and especially by the energy-oriented Technocrat movement, which advocated replacing politicians and business leaders with scientists and engineers. He would go on to hold important positions at Shell Oil and the United States Geological Survey (USGS).

Hubbert's logic for peak oil was simple: Oil cannot be produced unless it is found. Because the finding of oil had followed a roughly normal curve, with a U.S. peak in the 1950s and a global peak in the 1960s, he reasoned that production must eventually follow the same normal-shaped curve. The logic was beautiful and consistent with theoretical and empirical data.

Hubbert's predictions were met with anger and disbelief. Leading the charge were

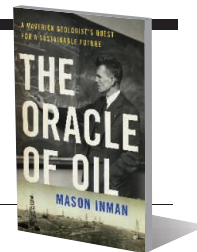


Hubbert "led the earth sciences kicking and screaming" from a largely qualitative field to a predictive science, declared the National Academy of Sciences in 1991.

Texas oilmen. Inman recounts a particularly tense meeting in which Hubbert confronted a handful of industry executives about their oil estimates, which he considered unrealistically optimistic. Richard Gonzalez of Humble Oil gave him "one of the dirtiest looks I've ever seen," Hubbert later recalled. "I think that guy could gladly have knifed me." The oil industry was joined by a number of economists who had great faith in the market to resolve any shortages.

But Hubbert's most consistent critic was Vincent McKelvey, a fellow geologist at USGS. Inman relates their encounters with relish. In 1961, Hubbert invited McKelvey to submit a report on the topic of nuclear fuels for the National Academy of Sciences'

The Oracle of Oil
A Maverick Geologist's Quest
for a Sustainable Future
Mason Inman
Norton, 2016. 429 pp.



Committee on National Resources. He was surprised to receive a much more exhaustive report several months later and was particularly troubled by the report's large oil discovery predictions. McKelvey was aggressive and confrontational when Hubbert followed up. His "mind was made up," Hubbert later recalled. "I mean, it was just a waste of time."

The main difference between McKelvey's estimates and Hubbert's much smaller predictions arose because McKelvey applied the "Zapp hypothesis" to plot and extrapolate the quantity of oil found per foot drilled over time. McKelvey assumed that the United States would continue to find oil at the previous rate of some 120 barrels of oil per foot drilled, which he used to estimate future production. Hubbert showed that the rate of discovery was actually declining.

Hubbert was ultimately vindicated when the production of the oil in the United States peaked in 1970 (2). It declined nearly every year until 2008, when new fracking technologies reversed the decline.

Where does this leave us today? The answer is not as unequivocal as I once thought. Global production of conventional oil has remained more or less flat since about 2005 (Hubbert's predicted "undulating plateau"), but new technologies have enabled the exploitation of low-grade source rocks in North Dakota and Texas and ever deeper offshore exploration.

This has led to a second, somewhat smaller, U.S. oil peak, which appears to be on the decline as well. However, these new reserves are much more expensive to harness, causing a continued decline in energy return on investment. In Hubbert's words, "when the energy cost of recovering a barrel of oil becomes greater than the energy content of the oil, production will cease no matter what the monetary price may be."

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10.1126/science.aaf2220

LETTERS

Edited by Jennifer Sills

Grant funding: Playing the odds

FEW SCIENCE POLICY issues are more important than the allocation of research funding. Although a 2015 Report suggested that peer review has some ability to prioritize applications (1), it is not clear that the best science is being funded (2). There is evidence of poor precision and pervasive bias in peer review (3, 4), as well as disparities in success rates based on seniority, race, and gender of the author (5–7). The current system is fairly effective in identifying the top 20% of applications (1), but fewer and fewer of those are funded (8). Selection of the best of the best resembles a lottery in its unpredictability (2), but one that lacks the benefit of being truly random, due to bias.

A modified lottery system could improve the fairness and efficiency of grant peer review. Given that reviewers are able to identify infeasible, poorly conceived, unoriginal, or otherwise seriously flawed applications, the first stage would use traditional peer review to do what it does best: create two pools of applications. The top 20 to 30% would be deemed meritorious, and the rest would be nonmeritorious. The second stage would use a lottery to select applications for funding from the meritorious pool. Applications deemed nonmeritorious would receive detailed critiques to allow applicants to revise and resubmit. Meritorious applications that make the lottery but are not selected for funding could remain in the pool for future lotteries, thereby saving both applicant and reviewer effort. (An alternative might be to give those who did not receive funding priority in the next round, but this would give the lottery some characteristics of a waiting list and quota system, which would have its own disadvantages.) As the process is implemented, policy details could be worked out to address issues such as how many lottery rounds applications will be allowed and whether those that consistently fail to obtain funding due to bad luck could be chosen for selective payment by program officers. Just as passively managed diversified stock portfolios that rely on random fluctuations of the stock market generally outperform active



A modified lottery could streamline grant-funding decisions.

management based on expert predictions (9), a modified lottery-based funding strategy would maximize the return on society's investment in science by distributing funding as broadly as possible. Moreover, a precise determination of the percentage of meritorious applications remaining unfunded would be a powerful tool to advocate for increased federal budgetary allocations. If lotteries can be used to select individuals for military service, housing, or the receipt of scarce medical resources (10), perhaps they can also help distribute research funding more fairly.

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Undermined by overhead accounting

AT MOST U.S. INSTITUTIONS, an overhead charge (or indirect cost) averaging 52% (1) is collected on all research expenditures, except for equipment. However, equipment is defined by science funders such as the National Institutes of Health (NIH) (2) and National Science Foundation (NSF)

(3) as an article of tangible non-expendable property that is useful for more than 1 year and has an acquisition cost of \$5000 or more per unit. This definition, which allows collection of overhead for equipment costing less than \$5000, likely costs U.S. scientists collectively millions of research budget dollars annually (4).

If a piece of equipment costs \$4999, the average effective cost to a grant is \$7598.48. If the price of the same item is \$1 more, then it costs only \$5000 (saving more than \$2500 on the grant). If a scientist is trying to decide between two identical pieces of equipment that cost \$3300

and \$5000, the scientist will be a better steward of research funds by purchasing the more expensive tool. The cheaper tool costs more on the grant and thus reduces the funds available for research. This perverted economic incentive encourages wasted money.

It is tempting to think that such small research expenditures are immaterial, given that routine scientific projects cost millions of dollars in total expenditures. However, purchases under \$5000 often represent the majority of nonlabor-related research costs for standard research projects. Some of these expenditures are for supplies, but much of the core equipment used every day in labs falls into this under-\$5000 category. A tiny fraction of overhead is normally returned to the department to be used for research, but the vast majority of indirect costs are used to subsidize administrative salaries and building depreciation (5), neither of which directly benefits research. On the contrary, these expenses often hamper research. The benefits administrators reap from overhead create a second perverse incentive: Administrators fight to maintain the arbitrary value of \$5000 as the definition of equipment (2, 3).

This problem is not new. However, now it is becoming even more damaging as it is effectively creating a de facto tax on the mass diffusion and development of free and open-source hardware (FOSH) for science. FOSH is hardware designed in the same way as open-source software; the designs are freely available for all to use and modify. FOSH is now growing rapidly because the costs of scientific hardware are generally only 1 to 10% of the cost of proprietary tools (6–9). Thus, a \$40,000 proprietary tool can easily be replaced by an equivalent \$4000 FOSH instrument, which will be penalized with an

indirect cost of \$2000. These savings are now possible because of digital manufacturing technology such as 3D printers, which enable both new science (10) and an increased ability to replicate scientific equipment (6–10). The relatively minor development costs of FOSH result in enormous returns on investment for scientific funding agencies, as tools are digitally reproduced thousands of times for the cost of materials (11). By removing the arbitrary capital cost of equipment, the effectiveness of research funding can be improved, and society's investments will pay larger dividends both directly and indirectly.

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Cultural costs of tropical dams

RECENT PIECES IN *Science* rightly call for greater examination of the environmental, political, and economic trade-offs of tropical dams. In his Feature news story "Power play on the Nile" (26 February, p. 904), E. Stokstad explores political uncertainties of the Grand Ethiopian Renaissance Dam. In their Policy Forum "Balancing hydropower and biodiversity in the Amazon, Congo, and Mekong" (8 January, p. 128), K. O. Winemiller *et al.* herald the potential detriment to one-third of the world's freshwater fish species by unprecedented hydropower dam construction. In his Letter "Tropical dams: To build or not to build?" (29 January, p. 456), P. M. Fearnside asks a fundamental question about current development. Assessments of impacts of dams on riparian human populations typically focus on economic issues related to community displacement, or food security risks from loss of land or fisheries. However,

riparian human populations stand to lose much more than land, food, and income.

Free-flowing rivers hold special significance in indigenous cultures. In the Amazon, the Shawi bathe in rivers, gathering strength from water carried down from mountains and ancestors (1, 2). The Peruvian Kukama believe that people who have drowned in rivers and whose bodies aren't found live in underwater cities, communicating with relatives through dreams or shamans (3). The Gumuz people of Ethiopia's Blue Nile Valley—living in the shadow of the Grand Ethiopian Renaissance Dam's construction—have described the river as a second God, providing everything they need for living; most cannot imagine life without the river (4). A legendary water-dwelling creature—Mokele-mbembe, or "one who stops the flow of rivers"—has captivated explorers and locals of the Congo Basin for centuries (5). In the Mekong, many indigenous people believe that ancestral or animal spirits can influence flow and quality of water, and fear of mysterious creatures has prohibited fishing in certain areas (6). Native people of northern Thailand engage in ceremonial practices to show respect and gratefulness to supernatural beings thought to influence water resources (7).

We need better understanding of the implications of tropical dam proliferation for riparian human populations. An assessment of human and water security (8) that includes not only economics, politics, and environment but also culture would more accurately capture the costs and benefits of hydropower development and influence decisions on new tropical dams.

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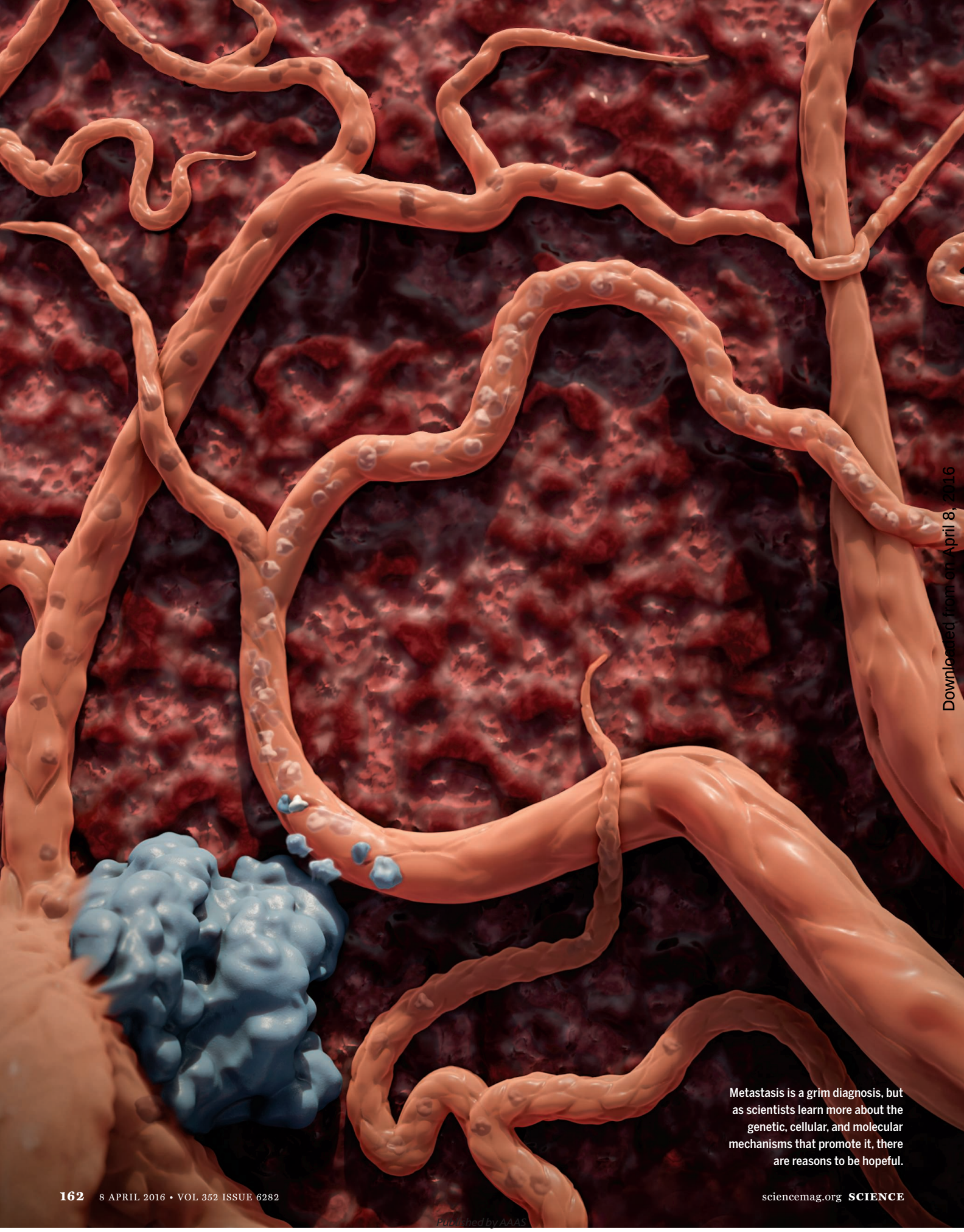
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Metastasis is a grim diagnosis, but as scientists learn more about the genetic, cellular, and molecular mechanisms that promote it, there are reasons to be hopeful.

METASTASIS

AN EVOLVING STORY

By Paula A. Kiberstis

As a medical diagnosis, metastasis instills fear. As a biological process, metastasis instills awe. A cancer cell's journey from a primary tumor to a metastatic site is a veritable obstacle course, and it is a miracle that any cell makes it to the end. After detaching from the primary tumor, a cancer cell must invade through local tissue, squeeze into blood or lymphatic vessels, survive the harsh conditions within those vessels, squeeze out of them, and begin growing in a new tissue, the microenvironment of which is often radically different from that of the primary tumor.

It was once thought that metastases are seeded by rare, rogue tumor cells endowed with specialized features that allow them to survive this perilous journey. As scientists apply new technologies to study metastasis, models of the process are evolving. Primary tumor cells do not always act alone but can invade as a group, circulate as a group, and seed growth at a distant site as a group. Upon arrival at that distant site, tumor cells may find that the microenvironment is already primed for their growth, thanks to signals sent in advance from the primary tumor via extracellular vesicles called exosomes. Comparative genomic analyses of primary tumors and metastases, although still works in progress, have already revealed considerable intertumor and interpatient variability in the origin, route, direction, and timing of metastasis. Somewhat disappointingly, there is no evidence to date of druggable "metastasis driver genes."

Nonetheless, there are reasons to be hopeful. Immune checkpoint inhibitors have shown encouraging results in patients with metastatic melanoma. Other immune-based therapies, such as drugs targeting neutrophils, are showing promise in preclinical models. Perhaps one day the research inspired by the awe will stamp out the fear.

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MALIGNANT MESSENGERS

Tumor cells may send out tiny vesicles that prime organs for cancer to spread

By Jocelyn Kaiser

One day, while poring over slides of mouse lung tissue for signs of cancer, David Lyden noticed some odd red specks. They were so small under an electron microscope that he first thought of viruses. But the animals had skin tumors that were prone to spread to the lungs, and the skin tumors had been dyed red. Lyden eventually concluded that the specks were tiny vesicles that had budded off from the skin tumors and found their way to the lungs—and that they functioned as the cancer's advance guard.

Vesicles called exosomes that bud off from tumor cells may help cancers invade new organs.



That observation 10 years ago sent Lyden's career in a new and controversial direction. The physician-scientist at Weill Cornell Medicine in New York City has since become convinced that a primary tumor—the first to grow in a person's body—spews protein- and RNA-packed vesicles known as exosomes into the blood that have a powerful impact in distant tissues. Far from being passive trash bags, as some have thought, the exosomes help cells from the primary tumor take root in other organs, or metastasize, the process that ultimately kills 90% of cancer patients.

In a series of provocative papers, he and his colleagues have injected exosomes from cultured tumor cells

into mice and observed striking effects on metastasis. They conclude that exosomes “prime” certain organs by making them more hospitable to primary tumor cells and “educate” other, nontumor cells to help the new cancer flourish.

Lyden's ideas run counter to the traditional view of metastasis, which is that cells shed from the primary tumor simply take root in distant organs and grow. “Most cancer biologists think the tumor cell dictates every part of the metastatic cascade,” Lyden notes. But he believes that exosomes prepare metastatic sites by changing tissue so it can nourish tumor growth long before cancer cells arrive.

“Tumor cells are pretty much an innocent bystander. They go to organs where pre-

ILLUSTRATION: V. ALTOUNIAN/SCIENCE

Downloaded from on April 8, 2016

metastatic niches are already established by tumor-secreted factors including exosomes," he maintains.

If Lyden is correct, tests for blood-borne tumor exosomes might provide a warning that a cancer is about to metastasize. His work also raises the prospect that targeting these vesicles with drugs could thwart the growth of metastatic tumors.

Lyden's ideas are "very interesting," says cancer biologist Raghu Kalluri of MD Anderson Cancer Center in Houston, Texas, who also studies the vesicles. But in his view, "It's an open book still. Nothing is completely proven about exosomes and metastasis"—much less the relevance to human disease and treatments. Indeed, some biologists say Lyden has stretched his conclusions beyond what his data support. They question his work on several grounds—for example, that injecting mice with a large number of exosomes from cultured cancer cells may not entirely reflect what happens in a living animal.

One Lyden collaborator says skepticism is a natural reaction. His proposal "is mind-boggling," says cancer biologist Mina Bissell of Lawrence Berkeley National Laboratory in Berkeley, California. "When someone is brave enough to make observations that are turning the field on its head, that's where the uncomfortableness comes in," she says. "People start bashing it."

EXTRACELLULAR VESICLES, membrane-enclosed packets released by many types of cells, were first noticed more than 30 years ago emerging from red blood cells. But interest picked up in the mid-1990s when immunologists realized these vesicles did more than help cells discard unneeded proteins—they could also help immune cells communicate (*Science*, 24 June 2005, p. 1862). Many can-

cer biologists have homed in on the smallest of these vesicles: exosomes, vesicles less than 100 nanometers in diameter, which are produced in abundance by tumor cells.

In the last few years, researchers have found that these tumor-derived exosomes can help a primary tumor invade neighboring tissue by transferring their cargo to adjacent, normal cells and influencing their behavior. Some studies have found that exosomes and larger vesicles can pass on oncogenic proteins that make the cells malignant in the first place.

Lyden, however, thinks exosomes also help tumors leapfrog to distant sites. Primary tumors metastasize by shedding cells into the bloodstream, but only a tiny fraction of those cells become established and grow in other organs. The successful colonists have help, Lyden showed about a decade ago. Building on evidence that bone marrow cells somehow nurture new tumors, Lyden's group gave mice transplants of green fluorescent-tagged bone marrow cells, then injected red-tagged melanoma or lung cancer cells under the animals' skin. The cells formed tumors and later metastasized to the lung.

The surprise was that green bone marrow cells appeared in the lungs several days before the metastatic tumor cells, and at exactly the same sites. Lyden concluded that the primary tumors somehow sent a message to the bone marrow cells to home in on future metastatic sites and produce proteins that would help blood-borne tumor cells take hold and grow, a phenomenon he dubbed the "pre-metastatic niche" in a widely cited 2005 paper in *Nature*. Even the liquid from cultured tumor cells seemed to recruit bone marrow cells, Lyden's group reported, suggesting factors within the liquid were conveying the messages.

Those factors, Lyden's lab eventually concluded, were carried by exosomes from primary tumor cells. In an attention-grabbing study published in 2012 in *Nature Medicine*, Lyden's team injected mice with one of three different treatments: exosomes from cultured, highly metastatic melanoma cells; exosomes from low-metastatic melanoma cells; or control particles. They then implanted highly metastatic melanoma cells under the animals' skin. Those given the exosomes from highly metastatic cells developed much more metastatic tissue.

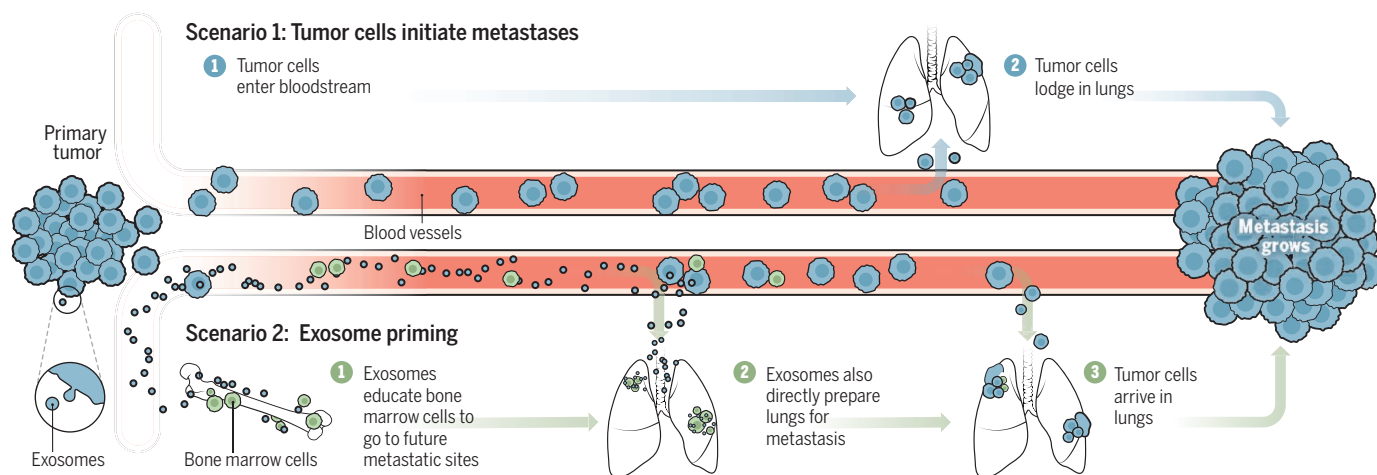
Lyden concluded that the tiny vesicles were creating his premetastatic niche, transporting an oncogenic protein called MET that prepared organs for tumor cells to take root and "educated" bone marrow cells to assist. Consistent with that scenario, Lyden reported in the same study that exosomes from the blood of cancer patients with advanced melanoma contained much higher than normal levels of MET.

Lyden and collaborators followed up with a similar paper last May in *Nature Cell Biology*, this time on pancreatic cancer, which tends to metastasize to the liver. Again they looked at how exosomes from cultured cells affected the spread of cancer in mice. They concluded that exosomes from pancreatic cancer cells educate certain liver cells to secrete molecules that create the fibrous environment conducive to the growth of tumor cells—this time via a protein called MIF.

Then last November in *Nature*, Lyden's team published an even more radical study, arguing that exosomes actually dictate which tissues a cancer will spread to. Exosomes carry integrins, the sticky proteins that cells use to interact. Lyden's group reported that exosomes from different types of cancer cells carry different sets of integrins. These direct the exosomes—and subsequent metastasis—to specific organs.

Two takes on metastasis

According to the traditional view, cells from a primary tumor enter the bloodstream, then a few lodge in distant organs such as the lungs. These cells then grow into new tumors. An alternative view is that primary tumors send out tiny, protein- and RNA-packed vesicles called exosomes that prepare distant sites for tumor cells to take root and recruit bone marrow cells to assist. This “pre-metastatic niche” nourishes the tumor cells that arrive later.



Exosomes from cancer cells that metastasize to the lungs, for example, were rich in two specific integrins. Giving these exosomes to mice with a type of breast cancer that normally spreads to the bones caused the cancer to spread to the lungs instead. These integrin “zip codes,” Lyden’s team suggested, could be used to predict where a patient’s cancer would metastasize. The team also showed in mice that blocking certain integrins curbed metastasis, pointing to a possible antimetastasis treatment.

CRITICS have several concerns. Lyden’s reliance on injections of cultured exosomes is one source of the skepticism. Some also question his evidence that certain proteins help exosomes prepare the ground for metastasis. For example, Lyden’s group used a short “hair-pin” strand of RNA (shRNA) to block a particular gene in tumor cells to curb their output of exosomes and, in other papers, limit production of MIF and integrins. But anonymous commenters on PubPeer, a website for discussing papers after publication, have complained that the researchers didn’t rule out the possibility that these shRNAs have “off target” effects, outside the exosomes, which could have skewed the results.

Kalluri and others also question his claim that the integrins on exosomes serve as zip codes for metastasis. They note that often exosomes from cell lines that metastasize to different organs have the same

integrins, so it’s not clear how the proteins could target exosomes to specific organs.

Lyden responds his group has done “multiple tests” to back up their experiments with shRNA. For instance, when they used peptides to block the binding of integrins, they saw the same effects. As for the role of integrins, he says critics have missed a key point: that it’s not only the presence of the integrin, but the amount that matters.

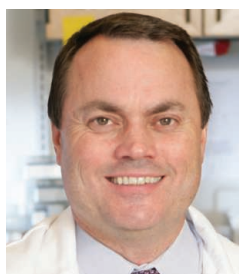
Recent work by other groups supports some parts of Lyden’s proposal. A study

More validation—or more questions—could come soon as part of the Reproducibility Project: Cancer Biology, an effort run by a nonprofit group to reproduce key experiments from 50 high-impact cancer papers. Lyden’s 2012 *Nature Medicine* paper is among them, and the replication results could be out later this year.

Even if Lyden’s claims hold up in animals, some are not convinced that the pre-metastatic niche—and the role of exosomes in shaping it—has much relevance for patients. “There is a disconnect between the animal studies and what is seen in the clinic,” says Joan Massagué, a metastasis researcher at Memorial Sloan Kettering Cancer Center in New York City. He explains that by the time most patients’ cancers are discovered and the primary tumor removed, a few metastatic cells have usually spread and lodged in

other organs, where they can lie dormant for years, then awaken and grow. In that case, Massagué argues, it’s not clear what role exosomes from a primary tumor that is no longer present would have.

But Lyden insists that exosomes—which he says metastatic tumors produce, too—are important to the spread of cancer even at that point. “If you could inhibit the role of exosomes, cancer progression can be thwarted and patients could live with cancer for a long time.” If that promise pays off, those little red dots he saw a decade ago could signal hope as well as a warning. ■



“Tumor cells are pretty much an innocent bystander. They go to organs where premetastatic niches are already established.”

David Lyden, Weill Cornell Medicine

published last May in *Cell* for the first time followed exosomes produced by primary tumors in living mice. Cancer biologist and imaging expert Jacco van Rheenen of the Hubrecht Institute in Utrecht, the Netherlands, and colleagues found that exosomes from highly metastatic tumors could make less malignant cells in distant organs more aggressive. “It is very much in line with the work of Lyden,” van Rheenen says. But he notes that his experiments don’t say whether the exosomes exert their effects even before primary tumor cells reach the sites of metastasis, as Lyden’s work suggests they do.

A collective route to metastasis: Seeding by tumor cell clusters

Kevin J. Cheung¹ and Andrew J. Ewald^{2*}

Despite decades of study, there are still many unanswered questions about metastasis, the process by which a localized cancer becomes a systemic disease. One of these questions is the nature of the tumor cells that give rise to metastases. Although conventional models suggest that metastases are seeded by single cells from the primary tumor, there is growing evidence that seeding requires the collective action of tumor cells traveling together in clusters. Here, we review this evidence, which comes from analysis of both experimental models and patient samples. We present a model of metastatic dissemination that highlights the activities of clusters of tumor cells that retain and require their epithelial properties.

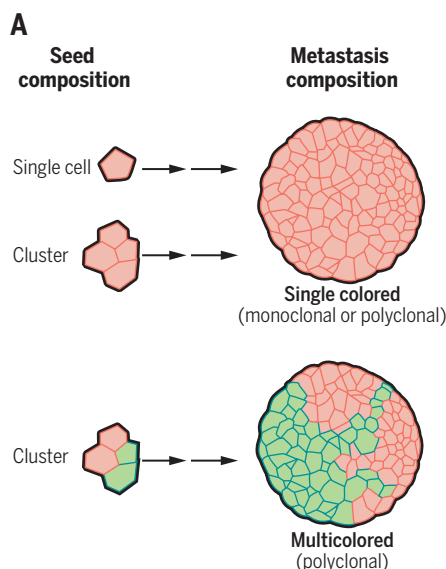
Metastasis is a complex, multistep process that requires cancer cells to detach from the primary tumor, migrate through adjacent tissue, access and travel through the vasculature, and then survive and proliferate in distant organs (1, 2). There is an added challenge for carcinomas because the adult epithelial cells from which they arise are normally polarized and nonmotile. Metastasis therefore represents a striking divergence from their homeostatic condition. However, epithelial tissues are highly dynamic and migratory during development and tissue repair (3), and epithelial cells can acquire mesenchymal molecular features in both developmental and disease states (4).

Current therapies are not sufficiently effective in treating metastatic disease, and so it is important that we determine the cellular and molecular features of the cancer cells that “seed” new tumors in distant organs. In this Perspective, we discuss recent work that supports a role for tumor cell clusters in metastatic seeding—an alternative to the conventional view that metastases are seeded by single cells from the primary tumor.

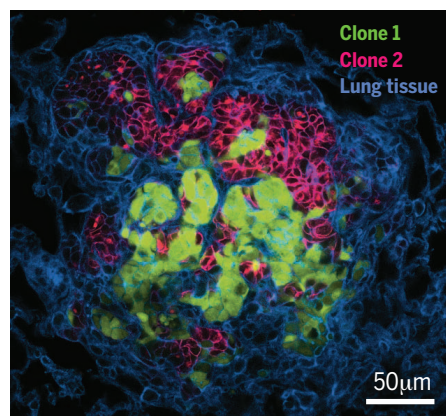
Tumor cell clusters and polyclonal seeding of metastases

The idea that tumor cell clusters contribute to metastasis can be traced back to the 1950s, when it was reported that blood samples from cancer patients contain both single and clustered tumor cells (2) and that tumor cell clusters can rapidly traverse the lungs in animal models (5). It was later shown that tumor cell clusters were more efficient than single cells were at producing metastases when injected intravenously into mice (2, 6). However, because metastasis is known to be inefficient (2), the data from these early studies are consistent with two very different possibilities: Metastases could arise from a less efficient process of seeding by abundant single

cells or from a more efficient process of seeding by rare clusters. Recent technological advances have revolutionized our ability to quantify circulating tumor cells (CTCs) and CTC clusters and to determine their molecular properties across diverse tumor types (7–10). In two such studies, the presence of CTC clusters was associated with significantly worse clinical outcomes as com-



B Multicolored lung metastasis



pared with the presence of single CTCs (8, 9). Their prognostic value is consistent with the hypothesis that tumor cell clusters make a distinct contribution to metastasis.

If the metastatic seed is a single cancer cell, then the resulting tumor will be clonal. Conversely, if the seed is a CTC cluster, then the resulting metastasis can be polyclonal from the start. Deep-sequencing analysis of tumors allows the reconstruction of evolutionary histories of cancer cell clones during metastatic progression. These evolutionary histories can be used to infer monoclonal versus polyclonal seeding. One recent study of prostate cancer patients revealed evidence for frequent polyclonal seeding from the primary tumor to secondary sites and the polyclonal spread of existing metastases to new sites in the body (11). This observation emerges in the context of an expanding literature on phenotypic and genotypic diversity within primary tumors and an increased appreciation of cooperative and competitive dynamics among cancer cell clones (12). These studies suggest that different clonal combinations in the cluster could have very different properties with respect to growth and/or response to therapy.

Polyclonal metastases could arise in two distinct ways: direct seeding by a multicellular cluster or serial accumulation of multiple single cells at a common site. Mouse models have proved valuable in distinguishing between these two mechanisms. A key feature of these experiments is the ability to establish primary tumors containing a mixture of cancer cells that express fluorescent proteins of different colors (9, 13, 14). Both single cells and cell clusters composed entirely of the same color will generate single-colored metastases (Fig. 1A). Conversely, clusters composed of more than one color will form multicolored metastases, which would provide direct evidence of their polyclonal origin (Fig. 1, A and B).

Three independent research groups have conducted studies of this design using different mouse models, and all have found multicolored metastases, which is consistent with the concept that polyclonal metastases can be seeded by tumor cell clusters (9, 13, 14). The first group established multicolored mammary tumors in mice and frequently observed multicolored metastases (9). When they established a single-colored tumor on one side of the mouse and a different

Fig. 1. Lineage analysis to identify the metastatic seeds in mouse models. (A) A primary tumor composed of cancer cells of two different colors can generate single cells, single-colored clusters, and multicolored clusters. Single cells and single-colored clusters will generate single-colored metastases. In contrast, multicolored clusters generate multicolored metastases. The number of different colors observed in a metastasis can therefore be used to infer the cellular properties of its seed. (B) Representative example of a multicolored lung metastasis in a mouse model of breast cancer [adapted from (14)].

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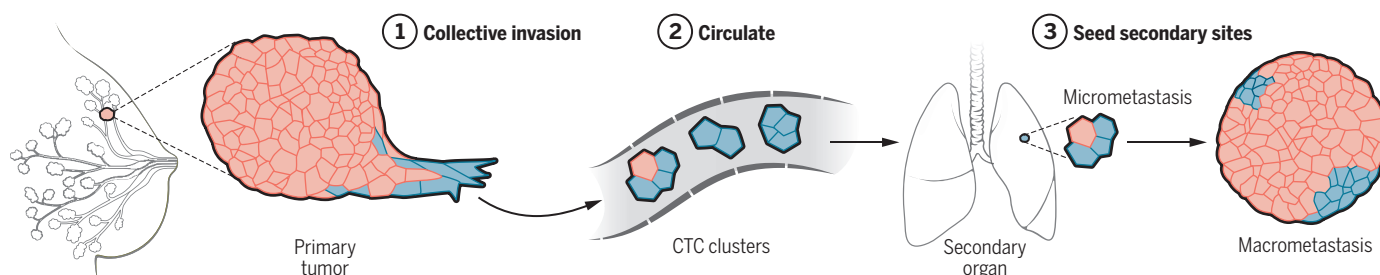


Fig. 2. A model for metastatic spread that is based on collective dissemination of epithelial tumor cell clusters. This model posits that primary tumor cells invade, circulate, and seed tumor growth at distant sites as collective units and that these activities require their expression of epithelial genes, such as K14. After arriving in the secondary organ (the lung), the predominantly K14⁺ seed (blue) from a primary breast cancer expands to form predominantly K14⁻ macrometastases (red). Further details are available in (14, 16).

single-colored tumor on the other side, the metastases were predominantly single-colored (9). This result suggests that aggregation of tumor cells in the blood or at the distant organ is inefficient and, therefore, that multicolored metastases arise from seeding by tumor cell clusters (9). The second group extended the lineage analysis concept to pancreatic cancer, documented polyclonal seeding by clusters in the mice, and observed strong differences in the extent to which a polyclonal seed expanded into clonal or polyclonal metastases in different organ sites (13). Studying spontaneous breast cancer in mice, the third group quantitatively related the extent of clonal mixing in the primary tumor with the frequency of detection of multicolored metastases, leading them to estimate that >97% of metastases arose from clusters (14). These three studies also provided direct evidence that clusters exhibit superior survival and colony-forming potential both in culture (14) and in vivo (9, 13, 14). Control experiments in which different color tumors were established in different locations in the mouse or different color cancer cells were injected intravenously at different times rarely or never yielded multicolored metastases. Therefore, all three studies provide data to support the concept that a multicellular cluster travels as a unit from the primary tumor to distant organs to seed polyclonal metastases (Fig. 2) (9, 13, 14).

Epithelial properties of the metastatic seed

The observations discussed above raise several intriguing questions, including how tumor cell clusters escape from the primary tumor and what their molecular properties are as they transit to the distant site. We will focus on breast cancer, where there has been substantial progress in understanding the molecular properties of tumor cells as they invade, disseminate, and travel to distant organs. Carcinomas can invade the adjacent tissue in a collective fashion, as groups of cancer cells that maintain at least some aspects of epithelial cell-cell adhesion (3). A recent three-dimensional analysis of the stromal border of human breast tumors detected frequent collective invasion and virtually no single cancer cells (15). These collective invasion strands could release small clusters of cancer cells to seed the metastatic process. A simple starting point for validating this model is to test for molecular

similarities between cancer cells in collective invasion strands and at later stages of metastatic spread. The cancer cells leading collective invasion were shown to have common molecular properties across subtypes of breast cancer, in both mouse models and in human breast tumors (16). These invasive cancer cells were characterized by their expression of molecular markers of basal epithelial differentiation, including cytokeratin 14 (K14), K5, P-cadherin, and p63 (16). Cells in this K14⁺ invasive state were observed in all stages of disseminative spread—collective invasion, local dissemination, CTC cluster, and micrometastasis—but were rare in the primary tumor and in macrometastases (14). These data suggest that there are distinct epithelial molecular programs driving tumor cell growth and dissemination (Fig. 2). Gene expression in these invasive leader cells resembles that of a basal phenotype mammary stem cell (14). This is consistent with single-cell transcriptomic analysis of human metastatic breast cancer cells (17), which showed that disseminated cancer cells share a basal stem cell gene expression profile in micrometastases but give rise to macrometastases composed largely of luminal phenotype cancer cells (17). Together, these studies reveal that changes in epithelial differentiation state accompany metastatic dissemination.

Epithelial cells are characterized by specific intercellular adhesion complexes. These junctions are often thought to impede motility; thus, it is conceptually attractive to think of metastasis as involving a transient or permanent loss of epithelial features, through a process such as the epithelial-to-mesenchymal transition (EMT) (4). However, recent studies have presented an alternative model in which epithelial adhesion complexes play an important role in metastatic tumor cells. These studies revealed that metastatic breast cancer cells retain the expression of epithelial cytoskeletal and adhesion genes such as K5, K8, K14, E-cadherin, and P-cadherin (14, 16). This concept is supported by the observation of the desmosomal adhesion protein plakoglobin in CTC clusters (9) and in the requirement for plakoglobin (9), E-cadherin (18), and K14 (14) in various experimental models of breast cancer metastasis. Knockdown of plakoglobin induced disaggregation of cancer cell clusters and compromised their efficiency in metastasis formation (9), whereas loss of K14 led to loss of a broader

program of metastasis-related genes, such as AdamTS1, CARD10, and tenascin C (14). Furthermore, the highly invasive K14⁺ cells in a mouse mammary tumor were enriched for the genes that constitute both a major epithelial cell-cell adhesion complex, the desmosome, and a major epithelial cell-matrix adhesion complex, the hemidesmosome (14). Thus, the epithelial cell adhesion machinery can be retained in disseminating cell clusters and can contribute to the metastatic process.

The role of epithelial genes in metastasis has precedent in developmental systems; migratory clusters of epithelial cells use mechanical signals through E-cadherin to coordinate their direction sensing and collective migration (19). Further studies are warranted to determine whether cancer cell clusters possess analogous migratory advantages during metastasis or whether their superior metastatic potential is solely attributable to enhanced survival. The retention of epithelial features in breast cancer clusters is also consistent with a recent demonstration that breast cancer cells do not require EMT to accomplish metastasis (20). Although the data clearly support the concept that epithelial-phenotype clusters can seed polyclonal metastases, it seems unlikely that this is the only mechanism of metastatic spread. Mesenchymal CTCs and CTC clusters exist in human patients, and the relative frequency of epithelial- versus mesenchymal-phenotype CTCs within a patient can shift with disease progression and cancer therapy (10). It remains an important future challenge to distinguish the relative frequency with which epithelial-phenotype versus mesenchymal-phenotype tumor cell clusters function as metastatic seeds.

Outlook

A number of studies in both preclinical models and patient samples have converged on the theme that tumor cells can metastasize collectively as clusters. Cancer cell clusters can retain and require epithelial gene expression and can transition between distinct epithelial differentiation states to accomplish the proliferative versus migratory components of metastasis.

At least three questions need to be addressed in this rapidly evolving field. First, what is the biological basis of metastatic seeding by tumor cell clusters? The answer to this question will require a deeper understanding of the mechanisms driving the enhanced metastatic efficiency

of clusters relative to single cells and molecular insights into how cell clusters coordinate their migration and differentiation through the many steps of metastatic spread. It will also be important to understand how clusters interface with stromal populations in the primary tumor, in the circulation, and in distant organs (1). Second, are the cellular and molecular properties of the metastatic seed the same across different tumor types and metastatic sites, or do they vary? Addressing this question will require lineage analysis techniques to quantify the fraction of single-cell versus multicellular seeds and extensive CTC and CTC cluster analyses in order to determine their molecular properties. Single-cell sequencing and proteomics will also be necessary to characterize the genotypic and phenotypic heterogeneity of the cancer cells within these clusters, both in the circulation and as they expand in different organ sites (12). Last, what are the therapeutic implications of tumor cell clusters? Clusters present a potential challenge because they could contain tumor cells with different drug-resistance properties. Cluster organization itself may also influence therapeutic response. On a more positive note, tumor cell clusters also present potential opportunities for novel therapeutic strategies that target either their multicellular organization or the changes in epithelial differentiation that enable their spread through the body.

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REVIEW

Metastasis as an evolutionary process

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Therapeutic advances in oncology have not fully translated to the treatment of metastatic disease, which remains largely incurable. Metastatic subclones can emerge both early and late in the life of the primary tumor. A better understanding of the genetic evolution of metastatic disease has the potential to reveal differences in the therapeutic vulnerabilities of primary and metastatic tumors, shed light on the temporal patterns of and routes to metastatic colonization, and provide insight into the biology of the metastatic process. Here we review recent comparative studies of primary and metastatic tumors, including data suggesting that macroevolutionary shifts (the onset of chromosomal instability) contribute to the evolution of metastatic disease. We also discuss the practical challenges associated with these studies and how they might be overcome.

Despite recent advances in the treatment of cancer, metastatic disease remains largely incurable and the main cause of cancer-related deaths. Metastases are the end result of a multistage process that includes local invasion by the primary tumor cells, intravasation into the blood or lymphatic system, survival in circulation (hematogenous and/or lymphatic), arrest at a distant organ, extravasation, survival in a new environment, and metastatic colonization. Each of these steps relies on specific phenotypic features of the tumor cell, as well as interactions with the host microenvironment and the immune system (1, 2).

Genetic and epigenetic alterations acquired by primary tumor cells and metastases probably contribute to these phenotypes and host interactions. Studying the genetic similarities and differences between primary tumors and metastases has the potential to provide new insights into the biology of metastasis. It may also reveal differences in the therapeutic vulnerabilities of local and systemic disease and shed light on the temporal patterns of and routes to metastatic colonization—information that could ultimately improve treatment and suggest strategies for preventing metastasis. Here we review recent studies examining the genetic evolution of metastases. We first describe some of the methodological challenges associated with determining the clonal relationship between the primary tumor and its metastases. We focus particularly on the challenge posed by intra-tumor heterogeneity (the occurrence of multiple genetically and geographically distinct tumor cell subpopulations within a single tumor), which has been documented in most solid primary tumors (3). We then synthesize

and interpret the results acquired to date in this burgeoning research area.

Current models of the evolution of metastatic disease and challenges in distinguishing between them

There are two general models of metastatic dissemination: the linear progression model and the parallel progression model. Both models assume that the primary tumor and its metastases are clonally related, in that they derive from a common ancestral cell. The two models are distinguished principally on the basis of (i) the relative timing of the emergence of the metastatic precursor population in the primary tumor and (ii) the expected genetic divergence between the primary tumor and its metastasis. The latter (termed P-M genetic divergence) is the number of independent single nucleotide variants (SNVs) accumulated by the primary tumor and the metastasis after the appearance of the most recent common ancestor (Fig. 1).

In the linear progression model (4), the metastasis-competent clone arises late in tumorigenesis and disseminates just before clinical detection of the primary lesion (5). The degree of P-M genetic divergence is expected to be small, because the metastasis is seeded by the most advanced primary clone or subclone (Fig. 1, top right panel). In the parallel progression model, the metastatic clone or subclone disseminates from the primary tumor early, and both the primary tumor and the metastasis continue to evolve in parallel, resulting in substantial genetic divergence between the primary and metastatic lesions (Fig. 1, top left panel). The parallel progression model assumes that multiple waves of dissemination of metastasis-establishing clones occur long before the primary tumor is clinically detectable, and that genetic alterations that are required for metastatic colonization evolve outside the primary tumor (5).

Interpreting genomic data within these frameworks is problematic, given that neither model accommodates the subclonal complexity (intra-tumor heterogeneity) of the primary tumor or the inevitable P-M divergence that arises through

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the inherent genomic instability of tumor cells (6). In comparative studies of P-M pairs, spatially limited sampling of the primary tumor (e.g., by a single biopsy) incompletely resolves its clonal structure and can lead to erroneous inferences of P-M divergence. This concept is illustrated in Fig. 1. The apparent genetic divergence between the primary tumor (P) and the metastasis (M) would decrease if two or more regions of the primary tumor (P1 and P2), rather than only one (P1), were sampled. Sampling a third region of the primary tumor (P3) would potentially decrease the apparent P-M divergence further if, for example, this region proved to be the origin of the metastasis. Inferences related to the timing of the emergence of the metastatic precursor population in the primary tumor, which are critical for distinguishing between the linear and parallel progression models, are likewise vulnerable to sampling bias. Thus, in the left column of Fig. 1, which depicts early divergence of the

metastatic precursor population from the primary tumor, comparison of one primary tumor sample (P1) with the metastasis would support the parallel progression model, whereas data derived from comparison of three primary tumor samples (P1, P2, and P3) with the metastasis would support the linear progression model.

Several other caveats need to be considered when evaluating data from comparative genetic studies of primary tumors and metastases. Critically, the number and the breadth of clonal markers directly affect the inferred degree of genetic divergence in P-M pairs. Comparisons based on whole-exome sequencing (WES) or gene panels provide limited resolution relative to those based on whole-genome sequencing (WGS), and they may skew the data, because they assess only the coding regions of genes or known driver genes, respectively. Comparative studies in colorectal cancer (CRC) illustrate this point. In several studies that were

based on the profiling of 45 to 1321 cancer-related genes (7, 8), P-M concordance rates were found to be 80 to 85%, rising to over 90% if only recurrently mutated genes were included (9). When the same P-M pairs were compared at the whole-genome level, the degree of P-M divergence increased (7). This observation is unsurprising, considering that gene panels are informed by our knowledge of driver genes. Current approaches for identifying driver genes are heavily skewed toward identifying early (clonal) drivers, which, unlike late (subclonal) drivers, are almost always shared between a primary tumor and its metastases (unless they are lost during tumor progression). Additionally, comparisons of P-M pairs that are limited to a single type of somatic alteration [usually SNVs and small insertions and deletions (indels)] also present an incomplete picture, especially given that somatic copy number alterations (SCNAs) affect a larger fraction of the cancer genome than any other type of alteration does (10).

Additional factors that can confound the inference of the mode of metastatic spread include exposure to systemic therapy or known carcinogens. For example, systemic drug treatment of patients before tumor sampling can reduce or enhance clonal diversity in both primary and metastatic sites (11), thereby affecting estimates of divergence between the primary tumor and metastases. Chemotherapy in particular has an impact on the mutational landscape, as has been shown for temozolomide-treated glioma (12) and platinum-treated esophageal cancer (13). In addition, the number of mutations accumulated before malignant transformation directly affects the apparent degree of divergence between the primary tumor and the metastasis (14). Carcinogen-induced tumors have a high mutational burden, but most of the mutations are probably acquired before the onset of tumorigenesis. For example, ultradeep sequencing of cancer genes in sun-exposed normal skin revealed an ultraviolet-induced mutational burden similar to that seen in many cancers (15). Thus, in cancers such as melanoma (16, 17) or smoking-related lung cancer (11), a high proportion of mutations are shared by the primary tumor and its metastasis, potentially masking their actual divergence.

Having outlined these caveats, we next evaluate the data generated by recent WES- or WGS-based comparative studies of primary tumors and metastases, with a focus on the analyses of multiple spatially and/or temporally separated biopsy samples. We restrict our discussion to clonally related P-M pairs or groups.

Comparative genetic studies of primary tumors and metastases

Large-scale comparisons have been limited to cohorts of unpaired primary and metastatic tumors and are frequently based on a small number of known genomic alterations. In the past 5 years, much smaller but paired cohorts have been profiled in a less biased fashion across a number of solid tumor types. Although descriptive in

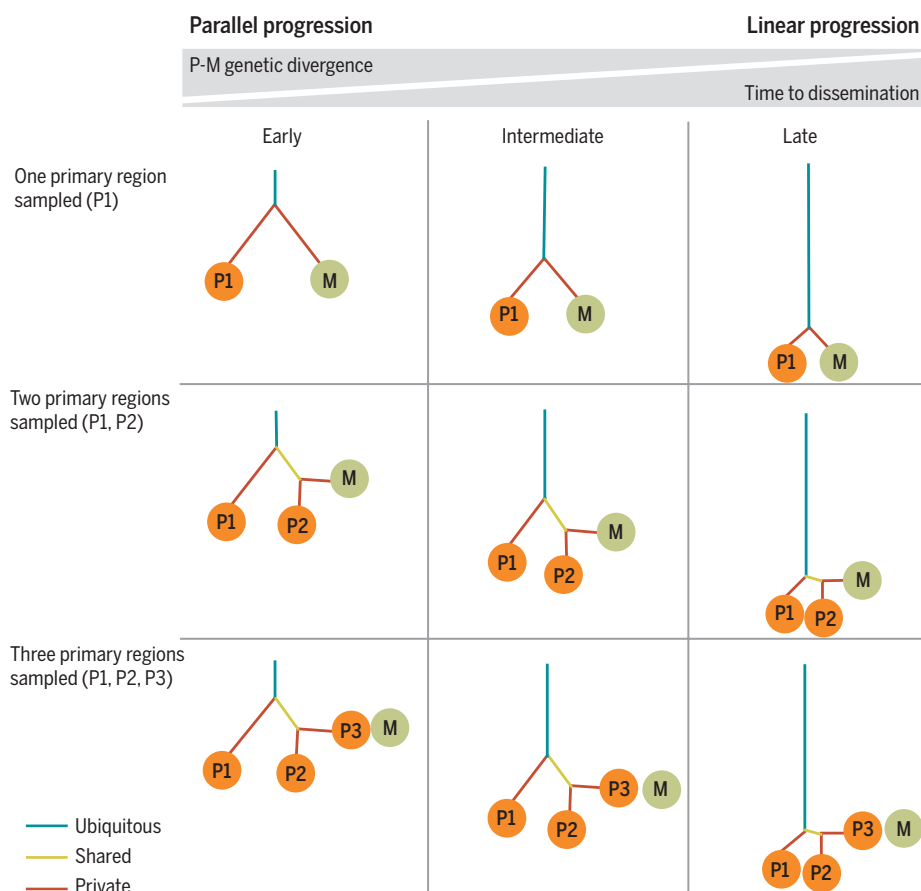


Fig. 1. Phylogenetic relationships in paired primary tumors and metastases, based on sampling of one, two, or three regions of the primary tumor. The phylogenetic trunk (blue) represents ubiquitous mutations, intermediate branches represent shared mutations (yellow), and terminal branches (red) represent private mutations. Metastases that disseminate from the primary tumor early show a substantial degree of genetic divergence (top left panel), whereas the late-arising metastatic subclone is very similar to the primary tumor (top right panel). Metastases can be identical to a subclone in the primary tumor (P3) or related to a subclone with evidence of additional alterations (P2), which is indicative of ongoing evolution. Limited sampling of the primary tumor can give the illusion of genetic divergence (top left versus bottom left panel) by failing to detect the metastasis-driving subclone in the primary tumor.

nature, these reports have provided unprecedented biological insights, and we therefore review them in detail.

Colorectal cancer

Genotype-based studies in CRC have consistently shown high levels of P-M concordance (18), leading some researchers to conclude that CRC conforms to the linear model of metastasis evolution. In the first prospective multiregional sampling study of CRC, Kim *et al.* (19) WES-profiled two to five primary regions and two to six liver metastases from five patients with microsatellite-stable metastatic CRC. Four of the five cases showed clear evidence of intratumor heterogeneity in the primary tumors, with up to 50% of coding mutations not shared by all the regions sampled. The primary tumor region that spawned the metastasis was identifiable in two cases, with enrichment for the metastatic clone evidenced by a higher cancer-cell fraction in the metastasis. "Private" genomic alterations (i.e., those that are found exclusively in one lesion) were observed in both primary and metastatic sites, although it is conceivable that these were in fact shared minor clones that evaded sampling or detection. Metastasis-private events included chromothripsis (a phenomenon in which one or a few chromosomes in a cancer cell bear dozens to hundreds of clustered rearrangements) and focal amplification of *MYC*. Chromothripsis was a shared event in another case that had evidence of additional SCNAs acquired in the rearranged chromosomal segments in the metastasis, indicating ongoing chromosomal instability (CIN). No alterations in known drivers of CIN or genome instability (GIN) were observed exclusively in the chromothripsis-containing metastasis; *TP53* clonal mutations were observed in all the cases studied. These data suggest that not all cases of CRC conform to the linear model of progression and raise the possibility that CIN plays a role in metastasis.

Breast cancer

A WES comparison study of a basal-like breast cancer primary tumor and a metachronous brain metastasis (20) revealed 48 SNVs that were shared by the primary tumor and the metastasis and only two SNVs that were private to the metastasis. Greater than 90% of the SCNAs detected in the primary tumor were propagated in the metastasis, whereas ~80% of SCNAs in the metastasis were not shared by the primary tumor. Because these additional SCNAs were also observed in a xenograft model derived from the primary tumor, it is likely that they were present in a minor subclone in the primary tumor that seeded the metastasis and was also selectively engrafted. This observation further supports the role of CIN in metastatic progression. In a recent multiregional profiling study by Yates and colleagues (21), metastatic subclones were identifiable in a primary basal breast tumor that gave rise to a lung metastasis and an estrogen receptor-positive primary breast tumor that gave rise to a lymph node metastasis. In that study, SCNAs

and *TP53* mutations were detected both clonally and subclonally across all histological subtypes of primary breast cancer. Through profiling of 52 single cells from a primary basal breast tumor and 48 cells from its liver metastasis, Navin and colleagues (22) found a high level of concordance at the level of SCNAs, which is consistent with limited P-M divergence or reseeding of the tumor by the metastatic cells (23) (the direction of travel is discussed further below). Only one report provided evidence of true parallel progression, with considerable P-M divergence observed in a case of lobular breast cancer. However, this inference was limited by select SNV profiling of the primary tumor and exclusion of SCNAs from the analysis (24).

Pancreatic cancer

Two studies in pancreatic cancer based on SNV (25) and SCNA (26) data traced the metastatic subclones to specific regions of the primary tumor. Yachida and colleagues found that most deleterious mutations and CIN were accumulated in the primary tumor, with limited private alterations evident in the liver, peritoneal, and lung metastases, suggesting that the lethal

"...evidence from these studies indicates elements of both linear and parallel metastatic progression within specific tumor types and even within individual cancers."

subclone emerged late (25). Through modeling these data, the authors estimated an average of 7 years between the birth of the cell that gave rise to the parental clone and the seeding of the index metastasis, representing a substantial window of opportunity to prevent metastatic disease (25). In their analysis of primary pancreatic tumors and lung, liver, and peritoneal metastases, Campbell and colleagues (26) reported that structural rearrangements often occurred as shared events, indicating that these alterations occurred early in tumorigenesis. However, a varying degree of P-M divergence was noted, owing to ongoing evolution in both primary and metastatic lesions; the data were consistent with parallel progression involving known oncogenes (amplification of *KRAS* and *MYC*) in some cases.

Renal cell carcinoma

Spatial and temporal resolution of a primary renal cell carcinoma (RCC) (27) revealed the origin of its metastatic subclone, which continued to evolve after dissemination to the chest wall and perinephric fat. Parallel evolution of distinct mutations in *SETD2* (coding for a histone-

lysine *N*-methyltransferase) and convergent SCNAs were observed in two cases with chest wall and liver metastases, respectively (28). Parallel evolution of distinct mutations in *PBRM1* (coding for a component of the SWI/SNF-B chromatin remodeling complex) was reported in a primary RCC and its brain metastases (29). Similar patterns were observed in four CRC cases with respect to distinct mutations in *TP53* (7). These findings indicate a strong selective pressure for (in)activation of these genes, both locally and systemically.

Prostate cancer

Several recent studies in prostate cancer P-M pairs have been particularly illuminating in terms of the wide range of patterns of metastatic progression. Comparative analysis of 333 primary prostate cancers (represented by single biopsies) (30) and an unrelated cohort of 150 bone and soft tissue metastases from castration-resistant prostate cancer (31) found the mutational and SCNA burden to be significantly higher in the metastases than in the primary tumors. These findings are consistent with the observation that patients with a high burden of SCNAs have an increased risk of relapse (32). Overall, the metastatic samples showed more frequent alterations in the *AR* gene (encoding the androgen receptor), *TP53*, *RBI*, the lysine *N*-methyltransferase genes *KMT2C* and *KMT2D*, and genes implicated in the DNA repair and phosphatidylinositol 3-kinase (PI3K) pathways. These results suggest substantial P-M divergence in prostate cancer, but they could also reflect the failure to sample the minor subclone in the primary tumor that spawned the metastasis. Accordingly, WGS analysis of multiple metastases that arose 17 years after resection of a primary prostate cancer traced their origin not to the bulk of the tumor but to a 2-mm low-grade region of it (33).

Using a combination of WGS and deep targeted sequencing, Hong and colleagues (34) profiled multiple regions of four primary prostate cancers and their associated metastases. Two primary tumors showed clear evidence of branched evolution. In the first case, the metastatic subclone was detected in the primary tumor, with additional variants involving *TP53*, *AR*, and *MSN* (coding for moesin) detected exclusively in the metastasis; in the second case, the primary tumor was largely unrelated to its metastasis, indicating early divergence of the metastasis-seeding clone and parallel progression in the primary tumor and metastasis. Enrichment for mutations in *TP53*, *AR*, and *MYCN* was also observed in the metastases in these four cases, as well as in an additional 19 cases in the extension cohort (34). Where paired primary lesions were available, *TP53* mutations were detected in minor subclones of the primary tumor. Taken together with the data from CRC studies, these findings offer further evidence that the acquisition of *TP53* mutations is associated with the expansion of subclones with metastatic potential. Microsatellite instability (MSI) and a mutation in *BRC42* were also observed as

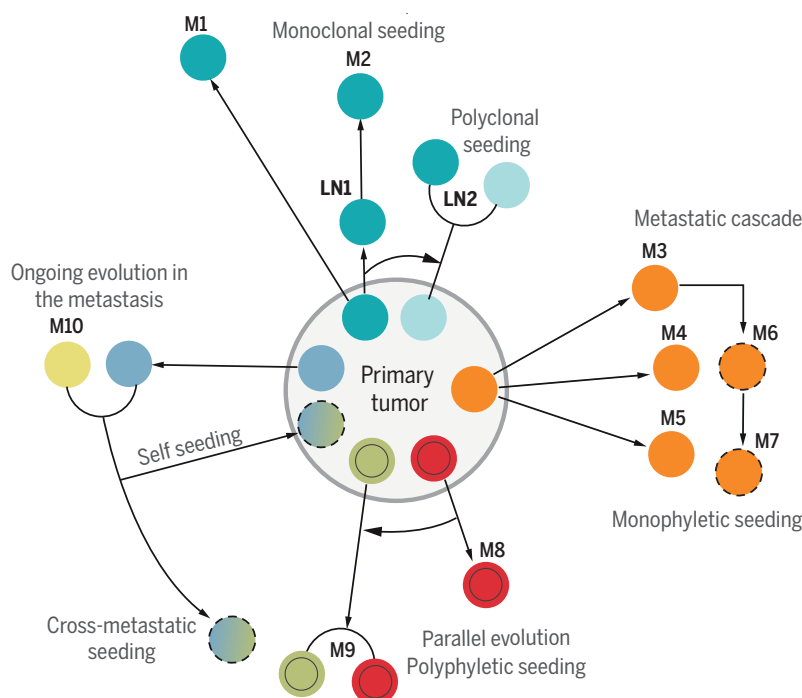


Fig. 2. Metastatic spread can take place through multiple routes and in different directions.

The primary tumor (P) is shown in the center of the diagram, with different colored circles representing distinct subclones. Lymph nodes can be seeded by single clones (LN1) or multiple clones (LN2). Lymph nodes can seed systemic metastases directly (M1), or the same primary subclone can seed a systemic metastasis (M2) in parallel to seeding the lymph node. A subclone other than the one that seeded the lymph node can seed individual systemic metastases in parallel (M3, M4, and M5; monophyletic metastases). Alternatively, it can seed just one metastatic site (M3), which in turn can seed other metastases (metastatic cascades; M6 and M7). Metastases can also be polyphyletic, with distinct subclones seeding different metastases (for example, M1, M3, and M8). Metastases can undergo parallel evolution, indicated by the double circles (M8 and M9). Metastases can continue to evolve after they have disseminated from the primary tumor, represented by the dual colors in M10. These metastases can then potentially re-infiltrate the primary tumor or surgical bed, a process called self-seeding. Cross-metastatic seeding can also occur, resulting in complex subclonal mixtures in the metastases themselves (M10→M9).

metastasis-exclusive in two cases (34). Last, there was evidence of two separate waves of metastatic spread from the primary tumor to the metastasis, suggesting repeated or continuous metastatic seeding. This observation has important clinical implications. If the primary tumor has the capacity to repeatedly seed metastases, then its removal, even if metastatic disease is already established, could halt further metastatic progression. It is this removal of the reservoir of diverse metastatic clones that is postulated to contribute to the survival benefit associated with palliative resection of the primary tumor in some advanced cancer, as observed in RCC (35).

Gundem *et al.* (36) characterized the subclonal architecture of ten cases of prostate cancer in which primary tumors had multiple paired metastases. In cases where the origin of the metastases was identifiable in the primary tumor, it was always a minor subclone, and both the primary and metastatic tumors continued to evolve after dissemination. In some cases, multiple subclones with different degrees of divergence from the primary tumor gave rise to the metastases, indicating independent and

temporally separate seeding, as was observed by Hong and colleagues (34). *AR* gene amplifications were subclonal in 16 of 17 primary tumors, which is consistent with the development of secondary resistance to androgen deprivation therapy (ADT) (37); the sequential gains of the *AR* gene that were observed in some cases imply continuous pressure exerted by therapy that selects for alterations in this gene and/or pathway. A similar phenomenon has been observed in breast cancer. Treatment of a patient exhibiting *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha)-mutant metastatic breast cancer with a drug that inhibits the PI3Kα signaling pathway resulted in parallel evolution of six distinct *PTEN* mutations in the metastatic sites, which had progressed through therapy and which were sampled at autopsy (38). Thus, selective pressure from therapy can also play a role in shaping metastatic progression.

In a recent report, Zhao *et al.* presented 40 P-M WES analyses across 13 different tumor types, including 13 cases of lung cancer (39). A varying degree of P-M divergence was observed. Early

divergence of metastases, consistent with the parallel progression model, was detected in 11 cases. However, no clear correlation was observed between the degree of P-M divergence and tumor type.

Several conclusions can be drawn from these analyses. First, evidence from these studies indicates elements of both linear and parallel metastatic progression within specific tumor types and even within individual cancers (Fig. 1, middle column). Although it may not be important to assign the model of progression correctly, an estimate of the timing of metastatic spread from the primary tumor has important clinical implications. If metastatic dissemination occurs before the primary tumor is clinically detectable, then early surgical resection may fail to reduce the risk of metastatic disease in the future.

Second, notwithstanding the small number of cases included, the studies reviewed here offer support for the idea that a macroevolutionary shift (defined as large-scale genomic alterations, such as copy number changes and structural chromosomal rearrangements manifested as CIN) is often evident at metastatic sites. Increases in CIN have been observed in parallel to metastatic progression in cases of prostate cancer (31, 32), pancreatic cancer (26), breast cancer (20, 21), CRC, and RCC (27). CIN is linked to poor outcomes in cancer treatment (40) and to metastatic progression in mouse models of skin carcinogenesis (41) and pancreatic adenocarcinoma (42). Evidence of parallel evolution was observed for *TP53*, as well as for other genes that play a role in maintaining genome stability: *SETD2* through nucleosome stabilization, suppression of replication stress, and the coordination of DNA repair (43, 44) and *PBRM1* through promoting cohesion of chromatin at centromeres (45). In RCC, *SETD2* and *PBRM1* loss-of-function mutations were frequently subclonal, suggesting that they are preferentially inactivated later in the evolution of the primary tumor, triggering CIN. The observed relationship between CIN and/or GIN and metastatic progression is reminiscent of the progression from pre-invasive to invasive disease, which is linked to the onset of CIN; two examples are the progression of Barrett's esophagus to esophageal carcinoma (46) and the progression of melanoma in situ to invasive melanoma (47). Karyotypic abnormalities such as genome doubling and chromothripsis were observed as a late event in some lung adenocarcinomas (11) and a metastasis-specific event in CRC (19), respectively. MSI and a *BRCA2* deficiency were detected exclusively in the metastasis of two prostate cancer cases (34), suggesting that distinct forms of GIN can be associated with metastatic progression.

Genetic divergence between metastases: The route and destination of metastatic cells

Genetic approaches have also been used to determine the relationship between two or more metastases arising from the same primary tumor.

Such studies can ascertain whether primary tumors contain multiple metastatic lineages that disseminate independently of each other and whether metastases that spread via lymphatics or blood (the route of travel) or to particular organs (destination of travel) are underpinned by genetic differences.

The relationship between metastases can also be described using the terms linear and parallel. In one hypothetical scenario, the same subclone of the primary tumor can seed both a metastatic lesion within a local lymph node (LN) and a metastatic lesion at a distant site, such as the liver or brain (Fig. 2). It could do so in a linear ($P \rightarrow LN1 \rightarrow M1$ in Fig. 2) or a parallel and independent fashion ($P \rightarrow LN1$ and $P \rightarrow M2$ in Fig. 2). In another hypothetical scenario, the subclone that seeds the distant metastasis (M3 in Fig. 2) may be distinct from the subclone that seeded the lymph node. Differentiating these scenarios in reality has profound implications for patient management, not least in terms of the morbidity of lymph node dissection. It is widely accepted that the clearance of involved lymph nodes improves local disease control, but whether it influences systemic dissemination is less clear. In cases where systemic metastases are established through hematogenous spread, lymph node clearance should have no overall survival benefit; conversely, if involved lymph nodes are a gateway to distant metastases, then their removal should reduce the risk of dissemination. A small number of comparisons between paired lymph node and distant metastases have been reported. In a WES analysis of a primary breast cancer, its axillary lymph node metastasis, and the synchronous systemic metastases, Murtaza *et al.* showed that a minor subclone in the lymph node gave rise to the distant metastases (48). Using WGS, Haffner and colleagues (49) tracked the clonal evolution of a prostate cancer that relapsed 17 years after radical surgery. Liver, bone, nodal, and soft tissue metastases were not seeded by the involved lymph node that was removed at surgery, but rather arose from a minor subclone in the primary tumor and contained additional alterations, including *AR* amplification, which were probably therapy-selected. A comparative study of metastatic melanoma demonstrated that one subclone of the primary tumor seeded loco-regional (i.e., localized to the area of the primary tumor) and lymph node metastases, whereas another subclone seeded a brain metastasis; in contrast, in another patient with melanoma, the lymph node and brain lesions grouped together and were distinct from the loco-regional metastasis (16). In combination, these studies show that systemic metastases can occur via direct hematogenous spread, bypassing the lymph node; however, some involved lymph nodes can seed systemic metastases, presumably through lymphovascular shunts.

The same subclone in the primary tumor can seed multiple metastases in different organs; such metastases are termed monophyletic. The seeding of these metastases can occur in parallel ($P \rightarrow M3$, $P \rightarrow M4$, and $P \rightarrow M5$ in Fig. 2), in

which case the metastases share the parental clone but can each harbor additional private subclonal mutations. Metastases can also appear monophyletic as a result of a metastatic cascade ($M3 \rightarrow M6 \rightarrow M7$ in Fig. 2), in which one metastatic lesion gives rise to another, and thus they are all closely related. Experimentally, it is challenging to distinguish these two models, unless the primary tumor is profiled exhaustively and all the metastases are sampled (49). Data on multiple metastases from the same organ, including from the lung (26), skin (50), and brain (51), are consistent with metastatic cascades taking place within organ boundaries. When distinct subclones in the primary tumor seed metastases in different target organs in parallel (M2, M3, M8, and M10 in Fig. 2) and at different times, the metastases are termed polyphyletic. In one illustration of this, distinct subclones of a primary pancreatic

“Future large-scale comparative studies with longitudinal and spatial sampling and robust clinical annotation will be required for reliable documentation of the patterns of metastatic dissemination within and across tumor types.”

cancer that seeded metastases in the lung and abdominal wall were found to harbor distinct mutations in *PARK2* (52), indicating parallel evolution between metastases. In a study of multiple metastases across a range of tumor types, monophyly and polyphyly were not correlated with any particular tumor type (39).

Some tumor types have the tendency to metastasize to certain organs and not to others, a phenomenon termed organ tropism (53). An extreme example is uveal melanoma, which metastasizes preferentially to the liver. Epidemiological evidence supports a relationship between organ tropism and certain tumor genotypes; for example, *KRAS*-mutant CRC is more likely to metastasize to the lung than to the liver (54), and half of *HER2*-amplified breast cancers metastasize to the brain (51). However, it is not known how these or any other genomic alterations influence organ tropism within or across tumors. Brain tropism is of particular interest, both biologically, because of the presence of the blood-brain barrier, and as a clinical problem, because of the poor prognosis associated with the diagnosis of brain metastases. Brastianos and colleagues (29) used WES to examine 86 cases spanning a variety of primary tumors and single or multiple paired brain metastases. In about half of the cases,

clinically informative genetic alterations (those that present actual or potential therapeutic targets), including *CDKN2A*, *PTEN*, and *PIK3CA*, were exclusive to the brain lesions. These observations are in keeping with the previous reports of increased activation of the PI3K-AKT pathway in brain metastases in general (55–57), suggesting that therapies targeting this pathway could play a role specifically in the treatment of brain metastases.

Genetic divergence between metastases: The direction of metastatic spread

Another phenomenon that can obscure phylogenetic relationships in genomic studies of metastases is self-seeding (M10 in Fig. 2). Thus far, we have discussed the various routes of metastatic spread but have assumed that the spread is unidirectional—that is, primary tumor cells seed the metastases, and metastatic cells do not seed the primary tumor. However, self-seeding (23), a process by which metastatic cells can re-infiltrate their tumor of origin, has also been proposed. Hypothetically, this would make the primary and metastatic tumors appear to be closely related. Experimentally, self-seeding is difficult to differentiate from the conventional linear progression from primary tumor to metastasis, because both processes would result in limited P-M divergence.

A proof of multidirectional seeding was provided by a study of human prostate cancer (34): The recurrence at the site of the resected primary tumor was found to be divergent from the original primary tumor and was seeded by a clone derived from a bone metastasis (an example of reseeding of the primary tumor by the metastasis). Repeat sampling also revealed cross-metastasis reseeding (M10 $\rightarrow$ M9 in Fig. 2) in response to ADT—that is, a resistance-bearing subclone from one metastasis invading an originally therapy-sensitive metastasis (34). Self-seeding could account for the observations made by Gundem and colleagues (36) and Haffner and colleagues (49), in which the origins of prostate cancer metastases were traced to a very small subclone in the primary tumor. The alternative hypothesis is that the metastases described in these two studies disseminated from the primary tumors, evolved further at distant sites, reentered the circulation, and reseeded the primary tumors, resulting in the detection of a minor metastatic subclone in the primary tumor.

Clonal dynamics between metastases: Do metastatic cells compete or cooperate?

When metastases are seeded by single cells or clones from the primary tumor (LN1 in Fig. 2), a reduction in genetic diversity is observed; that is, there is a decrease in the number of observed mutations in the metastasis relative to the primary tumor (provided that all of the subpopulations in the primary tumor were characterized) (M2, M3, and M8 in Fig. 2). This is analogous to a bottlenecking event in evolutionary biology. Further evolution can occur in the

metastasis, resulting in subclonal mutations, but these are not shared with the primary tumor (M10 in Fig. 2).

Comparative studies, as well as profiling of unpaired metastases, indicate monoclonal patterns of seeding across many human cancers (19, 25, 26, 29, 34), suggesting that clones compete to metastasize. However, polyclonal seeding, in which multiple clones from the primary tumor seed the same metastasis, is also observed (LN2 and M9 in Fig. 2); this indicates that subclones might cooperate as well as compete to metastasize. Comparative genomic studies have found that polyclonal seeding results in primary and metastatic tumors sharing multiple subclonal populations (17, 36). Polyclonal seeding can occur in parallel (direct clonal cooperation) or in temporally separate waves (indirect clonal cooperation); for example, the initial clone can remodel the metastatic niche, making it attractive for additional clones to colonize later (16, 36). In an example illustrating polyclonal seeding, two subclones in a melanoma primary tumor that were characterized by distinct mutations in the catenin beta 1 gene *CTNGB1* were found to seed the same loco-regional metastasis (16), suggesting that parallel evolution may support polyclonal seeding. Similarly, two subclones that contributed to the polyclonal seeding of a lymph node in a castration-resistant prostate cancer harbored distinct alterations associated with ADT resistance: *MYC* amplification and a pathogenic *AR* substitution (M9 in Fig. 2) (36).

Evidence from mouse models supports the notion that metastasis can arise from the collective migration and colonization of cancer cells (58, 59) and that cell clusters can be more efficient than single cells at forming metastases (58). Murine models of small cell lung cancer have demonstrated polyclonal seeding of lymph node metastases (60) and how one subclone can endow another with metastatic capacity through paracrine signaling (61). Recently, using lineage labeling, Maddipati and Stanger (57) demonstrated the polyclonal origin of metastases in a mouse model of pancreatic cancer. Cells were shed from the primary tumor as multiclonal aggregates, rather than one cell at a time. Although all metastatic sites were seeded polyclonally, monoclonal or polyclonal patterns of metastatic outgrowth were site-specific. Whereas peritoneal lesions remained polyclonal, liver and lung lesions drifted toward monoclonality, presumably reflecting different selective pressures at these metastatic sites. These data suggest that both clonal competition and clonal cooperation play a role in the evolution of metastases. In practical terms, the human tumor data indicate that a single biopsy of a single metastatic site may not accurately portray the polyclonality of that particular metastasis or the overall diversity of metastases.

Conclusions

Evidence from most comparative genomic studies of primary tumors and metastases indicates elements of both linear and parallel metastatic progression (Fig. 1, middle column), even with-

in the same patient. The timing of active sampling and analysis of the primary tumor relative to the disease course could affect which model of progression is observed. Thus, the two models are not mutually exclusive and are part of a biological continuum. Temporal waves of early and late, multidirectional, monoclonal and polyclonal, and monophyletic and polyphyletic metastatic spread are observed across cancers and within individual cases. Overall, no clear relationship has emerged between the mode of metastatic progression (linear versus parallel, monoclonal versus polyclonal, or monophyletic versus polyphyletic) and the characteristics of the primary tumor, including tumor type and other clinical features. To date, there is little compelling evidence for the existence of specific metastasis-driving genes, with the exception of *TP53*. Mutations in *TP53* appear to be linked to expansion of the metastatic subclones in prostate cancer (34) and in a subset of CRCs (28), possibly because *TP53* dysfunction is associated with tolerance of chromosomal instability and rearrangements (62) and chromothripsis (63). In this regard, CIN is a genetic hallmark that is frequently identified in metastatic subclones. Thus, there appears to be a link between the onset of CIN and metastases, but for this link to be corroborated, it is essential that future comparative studies include analysis of somatic copy number and structural alterations, in addition to SNVs and indels assessed longitudinally through the disease course.

The multitude of phenotypes required for metastases would be hard to achieve in a microevolutionary stepwise manner (i.e., through small-scale genomic alterations, such as single nucleotide substitutions and small indels affecting single genes). It is conceivable that macroevolutionary leaps (large-scale genomic alterations) (64) could catalyze all the steps to metastases, especially in narrow time frames, such as in synchronous presentation of primary and metastatic disease.

Future large-scale comparative studies with longitudinal and spatial sampling and robust clinical annotation will be required for reliable documentation of the patterns of metastatic dissemination within and across tumor types. Inclusion of postmortem sampling will be critical, because this allows exhaustive sampling of metastatic sites; evidence from autopsy studies shows that subclinical metastases are very common (65). Liquid biopsies have been shown to reflect the clonal dynamics of primary and metastatic tumors (48, 66, 67) and are an important adjunct to tissue analyses. The results of such studies could illuminate the relationship between the mode of metastatic spread and important clinical characteristics. These characteristics include primary tumor size [considering, for example, that the risk of late recurrence is the same for small and large breast primary tumors (68)]; the timing of metastases [considering the wide range observed across tumor types, from early (non-small cell lung cancer) to late (melanoma, RCC, and breast cancer)]; and patterns of

metastatic spread [organ tropism and whether metastases are limited to a single organ (oligo-metastatic disease) or widespread]. The ability to anticipate the route, direction, and timing of metastatic spread could inform many aspects of surgical management, including the value of metastasectomy (the surgical removal of metastases), lymph node removal, and palliative resection of primary tumors for disease control; management of small tumor masses (renal or prostate); and individualized follow-up care for patients who have had surgery with curative intent. Lastly, such large-scale studies will have the statistical power to identify metastasis-permissive and metastasis-suppressive processes in the tumor microenvironment and the immune system, thereby creating therapeutic opportunities to limit this almost intractable facet of cancer medicine.

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REVIEW

Hypoxic control of metastasis

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Metastatic disease is the leading cause of cancer-related deaths and involves critical interactions between tumor cells and the microenvironment. Hypoxia is a potent microenvironmental factor promoting metastatic progression. Clinically, hypoxia and the expression of the hypoxia-inducible transcription factors HIF-1 and HIF-2 are associated with increased distant metastasis and poor survival in a variety of tumor types. Moreover, HIF signaling in malignant cells influences multiple steps within the metastatic cascade. Here we review research focused on elucidating the mechanisms by which the hypoxic tumor microenvironment promotes metastatic progression. These studies have identified potential biomarkers and therapeutic targets regulated by hypoxia that could be incorporated into strategies aimed at preventing and treating metastatic disease.

Tumor metastasis is a major challenge in the clinical management of cancer. Metastatic disease is responsible for more than 90% of all cancer-related deaths and is often associated with high patient mortality because it is difficult to treat surgically or with conventional chemotherapy and radiation therapy. Metastasis is a complex and dynamic process that selects for highly aggressive tumor cells that acquire the ability to disseminate from their tissue of origin, survive within foreign tissue microenvironments, and grow at distant sites. Recent studies have demonstrated that cellular and molecular constituents regulated by the microenvironment in both the primary tumor and distant tissue profoundly influence the propensity of tumors to metastasize.

Oxygen is a microenvironmental factor that controls developmental processes, as well as normal tissue homeostasis. At the cellular level, oxygen is required for oxidative metabolism, the generation of adenosine 5'-triphosphate (ATP), and cell survival. To maintain oxygen homeostasis, metazoan organisms use the hypoxic signaling pathway to facilitate oxygen delivery and cellular adaptation to oxygen deprivation (1). Hypoxia is emerging as a key microenvironmental factor in the regulation of metastasis. Here we review our current knowledge of the roles that hypoxia and hypoxic signaling play in metastasis and highlight recent advances within this field.

Hypoxic signaling

The hypoxia-inducible transcription factors HIF-1 and HIF-2 coordinate the adaptive cellular re-

sponse to low oxygen tensions by activating gene expression programs controlling glucose uptake, metabolism, angiogenesis, erythropoiesis, cell proliferation, differentiation, and apoptosis (1). Both initiation and progression of tumorigenesis are promoted by HIF signaling, and tumors use multiple strategies to activate this pathway.

Hypoxia, or low oxygen tensions, is a hallmark feature of the tumor microenvironment. It has been estimated that 50 to 60% of solid tumors contain regions of hypoxia and/or anoxia that arise as a result of an imbalance between

oxygen delivery and oxygen consumption (2). Within the tumor microenvironment, oxygen delivery is impaired because of abnormalities in the tumor vasculature, including distended capillaries characterized by leaky and sluggish blood flow (3). At the same time, oxygen consumption rates are high because of the demands of proliferating tumor

cells and infiltrating immune cells. Clinically, hypoxia is associated with HIF activation, metastasis, and resistance to chemotherapy and radiotherapy, as well as poor patient survival, indicating that hypoxia may contribute to tumor progression and resistance to therapy (4–6).

Hypoxia activates HIF signaling by promoting the protein stability of HIF- α subunits. Under normoxic conditions, prolyl hydroxylase enzymes (EGLN 1–3, also known as PHD 1–3) use oxygen as a substrate to hydroxylate key proline residues within the HIF-1 and HIF-2 α subunits (7–9). This hydroxylation event allows the von Hippel-Lindau tumor suppressor protein (VHL), the substrate recognition component of an E3 ubiquitin ligase complex, to bind to HIF- α and target it for proteasomal degradation (10–14). Under hypoxic conditions, PHD activity is inhibited, resulting in HIF- α stabilization and translocation to the nucleus. In the nucleus, HIF- α subunits dimerize with aryl

“Clinically, [tumor] hypoxia is associated with HIF activation, metastasis, and resistance to chemotherapy and radiotherapy, as well as poor patient survival...”

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hydrocarbon receptor nuclear translocator (ARNT) and bind to hypoxia-responsive elements in target genes to activate gene transcription by recruiting transcriptional coactivators including p300/CBP (15–17). Hundreds of genes are activated in response to HIF-1 and HIF-2; these factors allow cells to survive and adapt to low oxygen tensions, as well as confer changes in both and host that promote metastasis (Fig. 1).

In addition to activation by hypoxia, the PHD-VHL-HIF signaling pathway can be regulated by genetic events and other microenvironmental factors. The enzymatic activity of the EGLN 1–3 requires iron and 2-oxoglutarate to hydroxylate the HIF- α subunits. As a result, EGLN 1–3 activity and HIF- α stability are affected by iron availability and can be inhibited by Krebs cycle intermediates, including succinate and fumarate, which compete with 2-oxoglutarate (18–22). In tumor cells, mutations in the genes encoding succinate dehydrogenase (SDH) or fumarate hydratase (FH) cause the accumulation of cyto-

solic succinate or fumarate; this inhibits the activity of prolyl hydroxylases and leads to HIF stabilization under normoxic conditions [for a recent review, see (22)]. Additionally, PHD activity can be inhibited by reactive oxygen species (ROS) produced by the mitochondria; however, the mechanisms by which this occurs under hypoxia remain to be defined (Fig. 1) (21, 23).

As noted above, VHL regulates hypoxic signaling by controlling the ubiquitination and degradation of HIF- α subunits. Genetic mutations or epigenetic inactivation of VHL causes VHL disease, which is associated with constitutive activation of HIF-1 and HIF-2 and the development of highly vascularized tumors, including hemangioblastomas, pheochromocytomas, pancreatic islet cell tumors, and clear cell renal cell carcinoma (ccRCC). Renal cell carcinoma is a major cause of morbidity in VHL patients, owing to the ability of these tumors to metastasize to other tissue sites (24). In addition, most sporadic ccRCCs lose VHL function through genomic alterations (25).

Although genetic inactivation of VHL is thought to be an early event in the pathogenesis of ccRCC, recent work has identified mechanisms by which tumors further potentiate HIF activity to promote metastatic progression. By analyzing metastatic subpopulations of VHL-deficient ccRCC cells, Vanharanta and colleagues discovered that epigenetic modifications within prometastatic genes allow for enhanced expression of metastasis-associated HIF target genes (26). For example, loss of polycomb repressive complex 2 (PRC2)-dependent histone H3 Lys-27 trimethylation activates HIF-mediated chemokine (C-X-C motif) receptor 4 (CXCR4) expression to promote invasion and metastasis (26). These findings open new areas of research aimed at understanding how epigenetic changes at prometastatic loci are altered during tumor progression. Recent studies have also indicated that VHL activity in tumors can be regulated by WSB1, an E3 ligase that targets pVHL for ubiquitination and proteasomal degradation (27). WSB1 expression

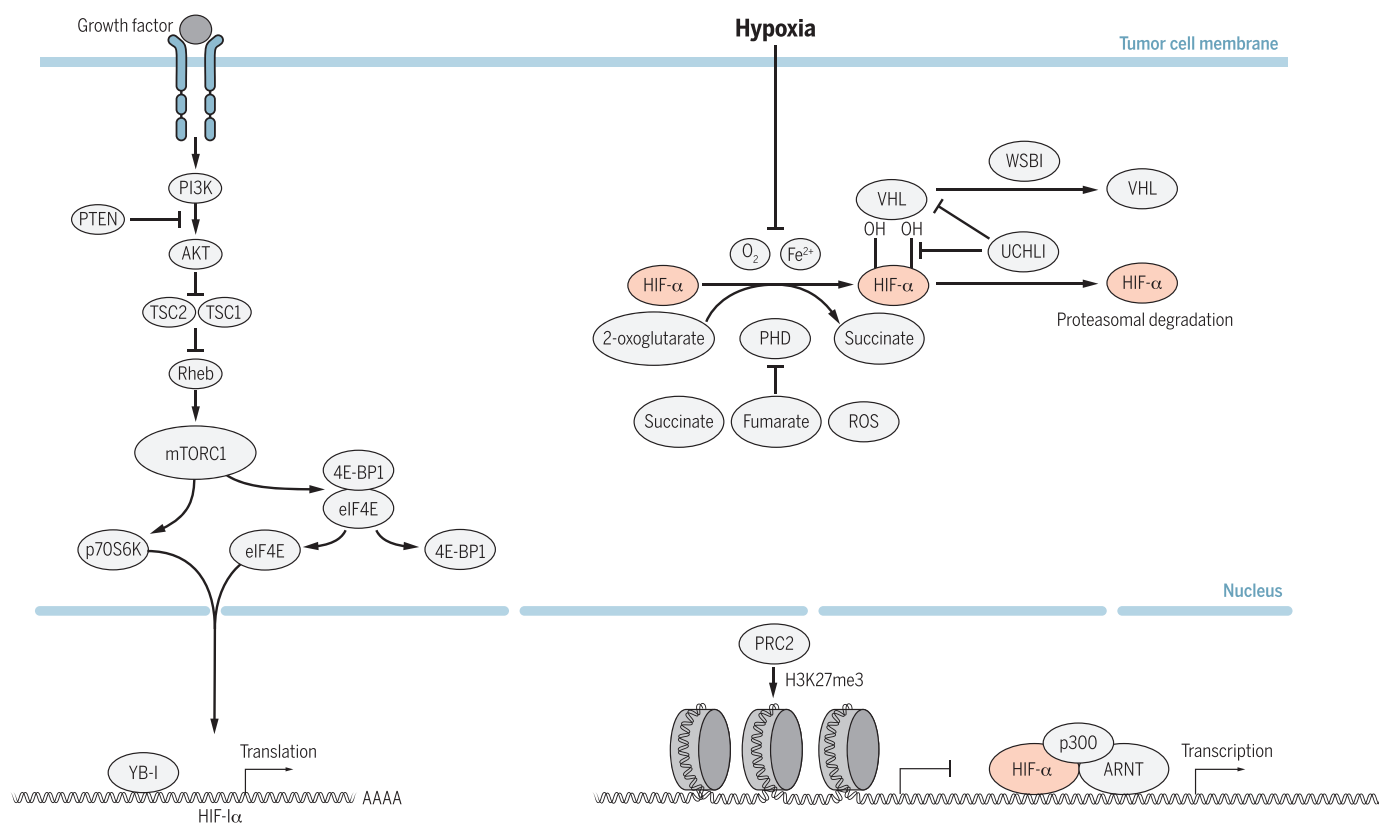


Fig. 1. Mechanisms of HIF-1 and HIF-2 activation in tumor cells. Hypoxia is a common mechanism of HIF activation in cancer. Under normoxic conditions, PHD enzymes (also called EGLN 1–3) utilize oxygen as a substrate to hydroxylate key proline residues located within the HIF- α subunit. This hydroxylation event mediates pVHL binding and subsequent ubiquitination and degradation by the 26S proteasome. Under conditions of hypoxia or loss of pVHL, HIF- α is stabilized and translocates to the nucleus, where it

heterodimerizes with ARNT and binds to hypoxia response elements (HREs) within regulatory regions of target genes. The HIF heterodimer activates gene expression at these sites upon cofactor (p300/CBP) recruitment. PRC2-mediated histone methylation can inhibit the activation of HIF target genes involved in metastasis. HIF activity can also be induced in tumor cells through mechanisms that enhance HIF- α production (TORC1, YB-1) or prevent its degradation (UCHL1, WSB1).

in tumor cells leads to HIF stabilization under normoxic and hypoxic conditions and correlates with metastasis in cancer patients (Fig. 1) (27).

Finally, hypoxic signaling can be activated by factors that promote HIF- α production or prevent its degradation. Both HIF- α mRNA transcription and translation are regulated by TORC1 activity [reviewed in (22)]. Inactivating mutations in phosphatase and tensin homolog (PTEN) or the tuberous sclerosis complex (TSC) complex, as well as activating mutations in phosphatidylinositol 3-kinase (PI3K) or AKT, lead to increased TORC1 activity that promotes HIF- α stabilization (22, 28–31). Additionally, activation of PI3K and AKT signaling through growth factor receptor signaling can promote HIF- α stabilization and activity under normoxic conditions (32, 33). HIF-1 mRNA translation can be enhanced under normoxic and hypoxic conditions through the binding of YB-1, an RNA and DNA binding protein; this leads to increased HIF-1 protein and prometastatic activity in sarcoma cells (34). HIF activity can also be promoted through the up-regulation of factors that prevent HIF- α degradation. Using an elegant screening approach to identify factors that would lead to HIF-1 transcriptional activity under normoxic conditions, Goto *et al.* recently identified the ubiquitin C-terminal hydrolase-L1 (UCHL1) as a HIF-1 deubiquitinating enzyme that promotes HIF-1 activity under normoxic and hypoxic conditions by preventing VHL-mediated degradation of HIF-1 (Fig. 1) (35). UCHL1 is associated with HIF-1 and distant metastasis in cancer patients, suggesting that UCHL1 may promote metastasis through HIF (35). In support of this concept, genetic inactivation of HIF-1 in UCHL1-overexpressing cells reversed the metastatic potential of these cells (35). In summary, these studies highlight the diverse mechanisms by which HIF signaling can be activated under hypoxic and normoxic conditions and may contribute to HIF activation during metastatic progression (Fig. 1).

Clinically, HIF-1 and HIF-2 are highly expressed in primary tumors and metastases. Immunohistochemical analysis of human primary tumor samples has revealed an association between high HIF-1 expression and metastasis in patients with gynecological, pancreatic, esophageal, lung, and prostate cancers (36–38). HIF-2 expression in primary tumors is also associated with distant metastasis in patients with small cell lung and breast cancers (39, 40). Moreover, increased HIF expression is often linked with increased patient mortality in a variety of human cancers (41). In experimental models, overexpression of HIF in tumor cells promotes metastasis (42, 43), whereas inactivation of HIF decreases the metastatic potential of tumor cells (43–47). Together, these clinical and experimental findings demonstrate an important role for HIF signaling in metastatic tumor progression. However, before this knowledge can be translated into safe and effective antimetastatic therapies, it is critical to understand how this pathway is used

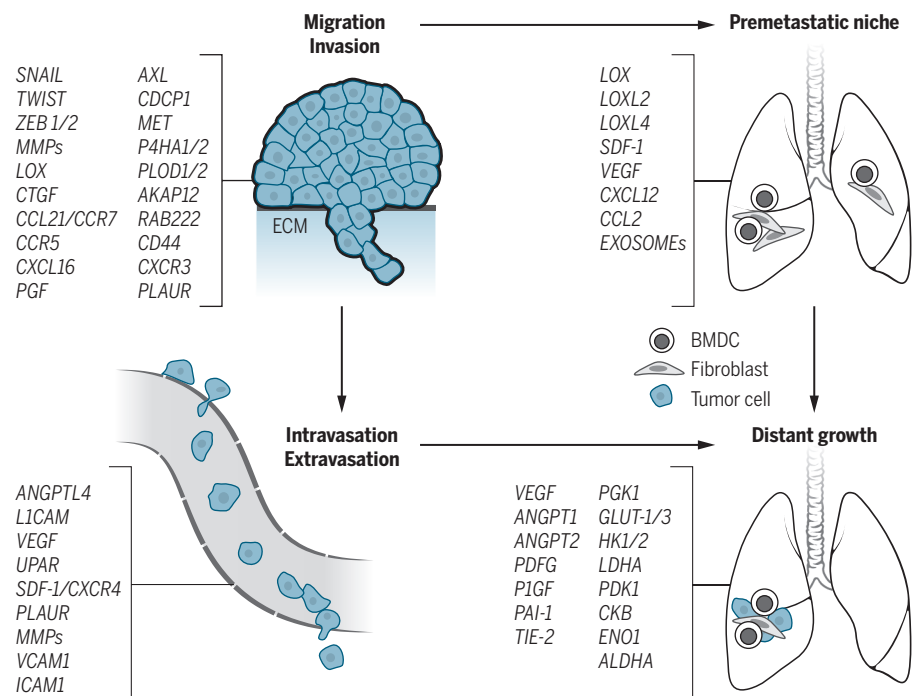


Fig. 2. HIF signaling regulates multiple steps within the metastatic cascade. Highlighted in brackets are direct target genes of HIF that promote each step of metastasis (to the lung, in the example shown). ECM, extracellular matrix; BMDC, bone marrow–derived cell.

by cancer cells and stromal cells to support metastasis.

Mechanisms of HIF-mediated metastasis

Hypoxia and activation of HIF signaling influence multiple steps within the metastatic cascade, including invasion and migration, intravasation and extravasation, and establishment of the premetastatic niche, as well as survival and growth at the distant site [Fig. 2; for recent reviews, see (48, 49)]. Here, we highlight recent studies that have revealed novel mechanisms by which HIF signaling promotes metastatic progression.

Hypoxic signaling and immune evasion

A critical step in metastatic tumor progression is the ability of tumor cells to evade immune attack. Tumor hypoxia is thought to promote the immunosuppressive phenotypes of both tumor cells and infiltrating immune cells. In preclinical models, respiratory hyperoxia (60% O₂) can promote tumor regression, reduce metastatic disease, and prolong animal survival (50). In these studies, respiratory hyperoxia was associated with a decrease in intratumoral hypoxia and an immunopermissive tumor microenvironment characterized by abundant CD8⁺ T cell infiltration and decreased regulatory T cell infiltration (50). Understanding the mechanisms by which hypoxia promotes immunosuppression is an active area of investigation and may have important therapeutic implications in the treatment of metastatic disease.

Hypoxia promotes tumor resistance to immune attack through several HIF-dependent

mechanisms. Normoxic and hypoxic stabilization of HIF signaling in tumor cells promotes resistance to cytotoxic T lymphocyte-mediated lysis (51–54). Hypoxia and HIF-mediated activation of autophagy in tumor cells also regulates natural killer (NK) cell-mediated antitumor responses; this occurs through the hypoxic degradation of NK-derived granzyme B in autophagosomes and the induction of inositol 1,4,5-trisphosphate receptor, type 1 (55, 56). Hypoxic signaling can promote resistance to T cell-mediated killing by increasing the expression of programmed death-ligand 1 (PD-L1) on tumor cells and myeloid-derived suppressor cells (MDSCs), and by enhancing CTLA-4 expression on CD8⁺ T cells, respectively [reviewed in (57)]. Additionally, hypoxic tumor cells evade innate immune recognition through the up-regulation of CD47, a cell surface molecule that interacts with signal regulatory protein alpha (SIRP α) on the surface of macrophages to block phagocytosis (58).

Hypoxia and HIF promote an immunosuppressive microenvironment by recruiting regulatory T cells (T_{regs}), MDSCs, and macrophages into the tumor microenvironment [reviewed in (57, 59)]. Hypoxic tumor cells recruit T_{regs} expressing CCR10 (CC chemokine receptor type 10) and NP-1 (neuropilin-1) into the tumor microenvironment through the secretion of CCL28, transforming growth factor-beta (TGF- β), and vascular endothelial growth factor [VEGF; reviewed in (57)]. Once in the tumor microenvironment, T_{regs} promote immune tolerance and angiogenesis, which supports metastatic tumor growth (60). Similarly, hypoxic tumor cells recruit myeloid

cells into the tumor microenvironment through the secretion of chemokines and cytokines, including CCL5, C-X-C motif chemokine 12 (CXCL12 or SDF-1), VEGF, and endothelins [ET-1 and ET-2; reviewed in (57)]. Hypoxic signaling in MDSCs and macrophages directly contributes to tumor progression by promoting an immunosuppressive phenotype and stimulating angiogenesis [reviewed in (57, 61–63)]. Moreover, hypoxia inhibits the effector functions of tumor-infiltrating lymphocytes largely through the accumulation of extracellular adenosine. Adenosine signals through A2A receptors on T cells to increase cyclic adenosine 3',5'-monophosphate, which inhibits T cell proliferation, expansion, and cytokine secretion [reviewed in (59)]. Collectively, these findings illustrate the diverse ways in which HIF signaling within tumor cells, MDSCs, and tumor-associated macrophages help establish an immunosuppressive tumor microenvironment.

HIF signaling in EMT, invasion, and migration

In the early stages of metastasis, tumor cells acquire invasive and migratory properties that allow them to exit the localized primary tumor mass and enter neighboring and distant host tissues. Tumor cells take advantage of many mechanisms to migrate and invade, including both individual and collective cell-migration strategies (64). In vitro tumor cell migration is associated with an epithelial-mesenchymal transition (EMT). EMT is characterized by changes in cell morphology, as well as cell-cell and cell-matrix adhesions. Cell-cell adhesions are mediated primarily by cadherin proteins expressed at intercellular junctions (65). Loss of E-cadherin allows cells to detach from their neighbors and

begin their migratory route toward the circulatory or lymphatic system to seek out new terrain. Reduced expression of E-cadherin is often observed in metastatic tumors, and work with experimental models has demonstrated that loss of E-cadherin is sufficient to promote metastasis of malignant cells (66).

Hypoxia or overexpression of HIF is sufficient to induce EMT and invasion in multiple cell types (42, 67–71). There are both direct and indirect mechanisms by which HIF signaling promotes EMT. A direct role for HIF in the regulation of key EMT transcription factors such as ZEB1, Snail, and Twist has been demonstrated through the identification of functional hypoxia response elements (HREs) within the regulatory elements of these genes (Fig. 2) (42, 72, 73). The HIF pathway also indirectly promotes EMT through a number of cell signaling pathways, including Notch (74, 75), TGF- β (76), integrin-linked kinase (77), certain tyrosine kinase receptors (48), Wnt (73), and Hedgehog (78, 79). We recently identified the AXL receptor tyrosine kinase as a novel HIF target driving EMT, invasion, and metastasis in VHL-deficient and hypoxic cancer cells (80). A growing body of literature supports an essential role for AXL in promoting metastasis within many tumor types, including breast (81), ovarian (82), and lung (83). Moreover, AXL inhibition has been reported to sensitize drug-resistant mesenchymal tumor cells to anticancer agents, including antimetabolic agents, epidermal growth factor receptor (EGFR) inhibitors, and PI3K inhibitors (83–86). Loss of AXL does not appear to have deleterious effects on embryonic development or normal adult tissue function in mice (87, 88). Moreover, biological inhibitors of AXL, such as soluble AXL decoy receptors, are spe-

cific and are not associated with normal tissue or hematologic toxicities in mice (82, 89). AXL thus appears to be a promising therapeutic target for the treatment of advanced cancer, and several inhibitors of AXL signaling are currently in preclinical and clinical development.

HIF signaling and the premetastatic niche

The late stages of metastasis are governed by the ability of disseminated tumor cells to colonize, survive, and grow within the distant tissue microenvironment. Over the past decade, experiments in mice have convincingly shown that formation of a tumor-promoting premetastatic niche in secondary organs is essential for the extravasation and growth of metastatic tumor cells at the distant site (90). The finding that the premetastatic niche is in part established by tumor-secreted factors has stimulated great interest in identifying the factors that govern tissue-specific metastasis for potential therapeutic targeting. The role of tumor microenvironmental factors such as hypoxia in the regulation of these secreted factors is likewise an area of intense investigation.

HIF signaling in the primary tumor contributes to the production of secreted factors involved in premetastatic niche formation (Fig. 2). In breast cancer cells, HIF signaling results in the increased expression and secretion of lysyl oxidase (LOX) and LOX-like proteins (LOXL2 and 4). These proteins modify the collagen matrix in the lung to recruit bone marrow-derived cells (BMDCs) that prime the lung for metastatic colonization. The BMDCs promote metastasis by producing chemokines that recruit tumor cells to the lung by mechanisms that include stimulation of tumor cell extravasation (44, 90–95). Recent

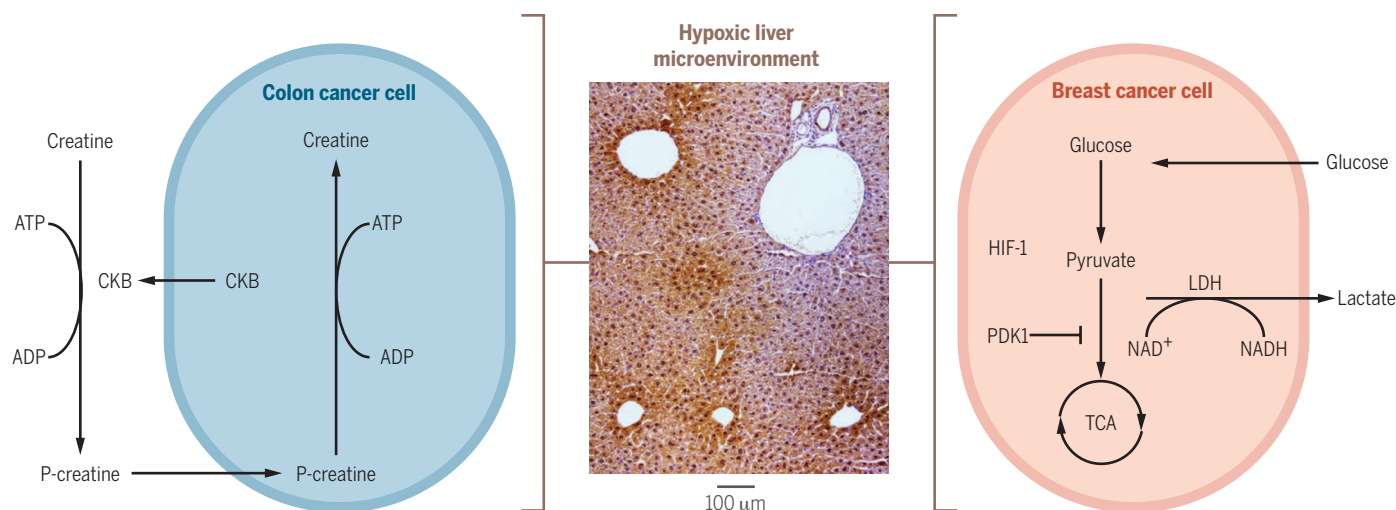


Fig. 3. Mechanisms of metabolic reprogramming in liver metastasis.

The liver microenvironment is hypoxic. Pimonidazole staining (brown) demonstrates areas of hypoxia in the liver (middle panel). Metastatic tumor cells enter hypoxic regions of the liver and must metabolically adapt to survive the metabolic stress associated with hypoxia. Disseminated breast cancer cells (right) metabolically adapt by increasing the expression of pyruvate dehydrogenase kinase 1 (PDK1) and promoting glycolytic reprogramming. Dissemi-

nated colon cancer cells (left) metabolically adapt by increasing the expression and secretion of creatine kinase brain-type (CKB). This enzyme controls the amount of rapidly mobilized high-energy phosphates by catalyzing the transfer of a high-energy phosphate group from ATP to the metabolite creatine, producing phosphocreatine. Under conditions of metabolic stress, such as hypoxia, tumor cells utilize phosphocreatine stores as a source of high-energy phosphate that can be transferred to ADP to generate ATP.

studies have shown that LOX is also a key factor in the establishment of the premetastatic niche in bone. Proteomic analysis of the hypoxic secretome in bone tropic MDA-231 human breast cancer cells compared to MDA-231 parental cells identified LOX as one of the most highly up-regulated secreted proteins in bone-tropic cells (96). Additionally, LOX expression is clinically associated with estrogen receptor negative (ER⁻) breast cancer metastasis to bone. In preclinical models, LOX expression in MDA-231 bone-tropic cells within the primary tumor is both necessary and sufficient to induce osteolytic bone lesions and cortical bone loss in immunocompromised mice even before the arrival of tumor cells. LOX regulates osteoclastogenesis, and high expression of LOX in primary tumors was found to be associated with the formation of osteolytic bone lesions in mice (96). These findings suggest that LOX may be an important biomarker to identify ER⁻ negative patients who are at risk for bone relapse. They also raise the possibility that LOX inhibition may prevent bone and lung relapse in breast cancer patients. Given that LOX also promotes tumor cell proliferation, invasion, and angiogenesis, LOX is an attractive therapeutic target for cancer therapy (97).

Another mechanism by which breast cancer cells condition the premetastatic niche is through tumor-lymphatic vessel cross-talk. Lymphatic vessels at the distant site support metastatic colonization in breast, melanoma, prostate, gastric, and colon cancer patients (98). Lymphatic endothelial cells (LECs) are a specialized endothelium that line lymphatic vessels and promote lymph metastases by actively recruiting tumor cells to the lymphatic vessels. LECs accomplish this by producing chemoattractants, including SDF-1 and CCL21, that bind to cognate receptors CXCR4 and CCR7, respectively, which are expressed by tumor cells (99). LECs within premetastatic organs are conditioned by tumor-derived factors to facilitate tumor cell recruitment, extravasation, and colonization (98). IL-6 secretion by the tumor activates signal transducer and activator of transcription 3 (STAT3) signaling in LECs localized within the lymph node and lung to promote (i) CCL5-mediated recruitment of CCR5-positive breast cancer cells into the lymphatic system and (ii) VEGF-mediated lung vascular permeability and lymph node angiogenesis (98). Interestingly, IL-6-induced VEGF expression in LECs is associated with the activation of HIF-1, indicating that tumor-derived factors may promote lymphatic metastasis at least in part through the activation of HIF signaling in LECs (98). Collectively, these studies demonstrate that hypoxia and HIF signaling mediates tumor-stromal cross-talk by activating the expression of circulating factors that can prime and direct metastatic growth.

An emerging area of interest is the role of exosomes in metastasis and whether hypoxia and HIF signaling might promote formation of the premetastatic niche by this mechanism. Exosomes are extracellular vesicles that regulate cell-cell communication by carrying and

transferring molecules including proteins, lipids, microRNAs, and mRNAs (100). Exosomes have recently been implicated in the establishment of the premetastatic niche. They are present at elevated levels in the serum of patients with cancer and are associated with advanced disease. Moreover, tumor-derived exosomes preferentially localize to sites of metastasis, including liver, lung, and bone marrow, where they promote vascular permeability and increase BMDC recruitment (101–104). However, the factors within the primary tumor that regulate exosome content and release remain largely unknown. Recent studies have shown that exosomes mediate hypoxia-dependent stimulation of angiogenesis in glioblastoma multiforme (GBM) through the activation of paracrine signaling in endothelial

“...hypoxia within the liver microenvironment selects for disseminated tumor cells that have the ability to metabolically adapt to the hypoxic stress.”

cells and pericytes (105). Interestingly, exosomes isolated from the plasma of GBM patients contain increased amounts of hypoxia-regulated proteins—including MMP9, MMP8, platelet-derived growth factor (PDGF), PAI1, and insulin-like growth factor-binding protein 3 (IGFBP3)—compared to exosomes isolated from plasma of sex- and age-matched controls (105). This work suggests that hypoxia can influence exosome cargo content to promote angiogenesis. Additionally, these findings suggest that the hypoxic signature of exosomes may serve as a noninvasive biomarker to predict the aggressiveness of malignant tumors. In addition to regulating exosome cargo content, hypoxia also promotes microvesicle shedding. Hypoxic cells up-regulate the small guanosine triphosphatase RAB22A in a HIF-dependent manner to promote microvesicle shedding, invasion, and metastasis (106).

HIF signaling and cellular growth and survival at the distant site

Successful metastatic colonization requires disseminated tumor cells to adapt to the microenvironment of the distant tissue, which can be very different from that of the tissue harboring the primary tumor. To illustrate this point, we discuss liver, a common metastatic site for colorectal cancer. The liver maintains overall energy balance by controlling carbohydrate and lipid metabolism. Within the liver, an oxygen gradient is established that provides boundaries for metabolic activities within the organ (Fig. 3). The partial pressure of oxygen in periportal blood is 60 to 65 mm Hg and in the perivenous blood it is 30 to 35 mm Hg (107). Because oxygen is an

essential electron acceptor for oxidative metabolism, hepatocytes that perform glucose or fatty acid oxidation are located in the aerobic periportal zone, whereas oxygen-independent metabolic functions such as glucose uptake, glycolysis, and fatty acid synthesis are predominantly performed by perivenous hepatocytes (108). Given that colon cancer cells metastasize to the liver through the portal circulation, Loo and colleagues (109) hypothesized that these cells would experience acute hypoxia and competition for glycolytic substrates. Using a HIF-1 transcriptional luciferase reporter mouse, they determined that colon cancer cells experience hypoxia early after hepatic dissemination. They found that the disseminated cancer cells increase their release of the enzyme creatine kinase brain-type (CKB), which helps them survive within the hypoxic microenvironment of the liver (109). CKB controls the amount of rapidly mobilized high-energy phosphates by catalyzing the transfer of a high-energy phosphate group from ATP to the metabolite creatine, producing phosphocreatine (110). Under conditions of metabolic stress, such as hypoxia, tumor cells use phosphocreatine stores as a source of high-energy phosphate that can be transferred to adenosine 5'-diphosphate (ADP) to generate ATP (110). Depletion of CKB increased caspase-mediated cell death in hypoxic tumor cells within the liver, suggesting that hepatic hypoxia is a barrier for colon cancer cells early in metastatic dissemination and that cells overcome this metabolic stress by generating ATP from phosphocreatine reserves (Fig. 3) (109).

Consistent with the concept that hepatic hypoxia and the associated glycolytic phenotype in periportal regions of the liver provide a barrier for metastatic colonization, Dupuy and colleagues observed that breast metastases to the liver are highly dependent on glycolysis for survival in comparison to bone and lung metastases (111). The metabolic reprogramming of liver-metastatic breast cancer cells was largely dependent on the HIF-1 target gene pyruvate dehydrogenase kinase (PDK1). PDK1 represses mitochondrial function by antagonizing the function of pyruvate dehydrogenase (PDH), a rate-limiting enzyme for pyruvate conversion to acetyl-coenzyme A and entry into the tricarboxylic acid cycle (112). Notably, PDK1 expression was elevated in breast cancer liver metastases compared to primary tumor specimens, further supporting the notion that PDK1 expression is particularly important for the growth and survival of breast cancer cells within the liver microenvironment. These findings suggest that breast cancer cells adapt to the hypoxic liver microenvironment through the activation of HIF signaling and glycolytic reprogramming mediated by PDK1 (Fig. 3). Thus, these studies demonstrate that hypoxia within the liver microenvironment selects for disseminated tumor cells that have the ability to metabolically adapt to the hypoxic stress.

Finally, HIF promotes late stages of metastasis at the distant site by stimulating angiogenesis. Like primary tumors, metastases require angiogenesis to support their growth. VEGF-A is

a proangiogenic factor produced by tumor cells that stimulates the recruitment and proliferation of endothelial cells, as well as promoting pericyte proliferation and migration (113). VEGF-A is a well-established HIF target, and its expression is induced by HIF signaling in both primary tumors and metastases (114).

Conclusions

Hypoxia and HIF-dependent signaling play an important role in metastatic tumor progression. Although hypoxia is an important factor that activates HIF signaling within tumors, recent studies have demonstrated that malignant cells use multiple strategies to enhance HIF signaling during progression by promoting HIF- α mRNA translation, protein stability, and downstream target gene expression.

The hypoxic tumor microenvironment influences both the early and late stages of metastasis. Hypoxic stress plays an important role in immune escape by promoting immune suppression and tumor resistance. Within the primary tumor, HIF-dependent gene expression controls EMT, invasion, migration, and angiogenesis to support the early stages of metastasis. Recent work has identified the receptor tyrosine kinase, AXI, as a critical mediator of HIF-dependent invasion and metastasis, as well as a potential therapeutic target for the prevention and treatment of metastatic disease. In addition, HIF signaling promotes the production of secreted factors such as LOX, LOX-like proteins, and exosomes to establish a prometastatic environment within the lung and bones of breast cancer patients. Hypoxia at the distant site also plays a substantial role in selecting for metastatic cells that can survive the metabolic stress associated with hypoxia. Metastatic breast cancer cells activate HIF signaling within the hypoxic environment of the liver to metabolically adapt to the glycolytic environment. Additionally, colon cancer cells adapt to the metabolic stress within the hypoxic liver by releasing CKB into the extracellular space to generate and import phosphocreatine as a source of ATP generation in this microenvironment.

In summary, recent studies have begun to highlight the diverse mechanisms by which hypoxia and HIF-dependent signaling promote metastatic tumor progression. As a result, a number of new biomarkers and therapeutic targets have been identified that could potentially be valuable for the detection and treatment of metastatic disease.

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RESEARCH ARTICLE SUMMARY

TRANSCRIPTION

Term-seq reveals abundant ribo-regulation of antibiotics resistance in bacteria

Daniel Dar, Maya Shamir, J. R. Mellin, Mikael Koutero, Noam Stern-Ginossar, Pascale Cossart, Rotem Sorek*

INTRODUCTION: Riboswitches and attenuators are cis-regulatory RNA elements (ribo-regulators), which in most cases control bacterial gene expression via ligand-mediated, premature transcription termination. Depending on the presence or absence of the specific ligand, the formation of a transcription terminator upstream of the gene causes transcription to abort prematurely, generating short unproductive transcripts. In response to changes in the metabolite concentrations, the structure of the ribo-regulator

is altered, destabilizing the terminator and allowing read-through into the gene, thus resulting in expression of the full-length mRNA. These ribo-regulators play central roles in bacterial physiology and virulence and have been used for synthetic biology applications as well as recognized as therapeutic targets for antibiotics.

RATIONALE: Despite the importance of riboswitches and attenuators, there is currently no experimental high-throughput method for the

discovery of such ribo-regulators across bacterial genomes. Furthermore, given a metabolite or ligand of interest, until now there was no efficient experimental approach to identify natural riboswitches or attenuators that sense and respond to it.

RESULTS: We developed term-seq, a method that enables quantitative mapping of all exposed RNA 3' ends in bacteria and allows unbiased, genome-wide identification of genes that are regulated by premature transcription termination.

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This method quantitatively measures the in vivo activities of all expressed ribo-regulators in a given genome simultaneously and under physiological conditions, thus enabling high-

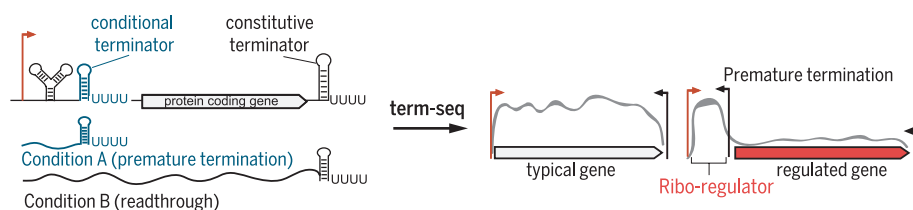
throughput discovery of ribo-regulators that respond to a metabolite of interest. Application of term-seq to the model bacteria *Bacillus subtilis*, *Listeria monocytogenes*, and *Enterococcus faecalis* detected the vast majority of known riboswitches as well as multiple previously unidentified regulators that function via conditional termination.

We demonstrate the utility of our approach by screening for ribo-regulators that specifically respond to small antibiotic molecules. We found that numerous antibiotics resistance genes, in both pathogenic bacteria and in the human microbiome, are regulated via termination-based ribo-regulators that allow read-through when the antibiotic is present in the cell.

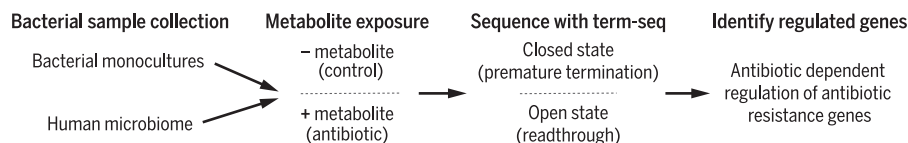
Focusing on *lmo0919*, one of the antibiotic-regulated genes we detected in *Listeria monocytogenes*, revealed that this locus confers specific resistance to the translation-inhibiting antibiotic lincomycin. In the absence of the antibiotic, transcription is terminated prematurely by the ribo-regulator. However, upon exposure to lincomycin, drug-inhibited ribosomes stall over a conserved three-amino-acid upstream open reading frame found within the ribo-regulator, thus triggering a conformational change in the transcriptional terminator and inducing the expression of the full-length mRNA that encodes the resistance gene.

CONCLUSION: These results describe a high-throughput method for ribo-regulator discovery in either bacterial monocultures or complex bacterial communities such as the human microbiome. Furthermore, they reveal a broad role for conditional termination in regulating multiple classes of antibiotic resistance genes in the human microbiome and provide a general tool for discovering riboswitches and attenuators that respond to specific metabolites of choice. ■

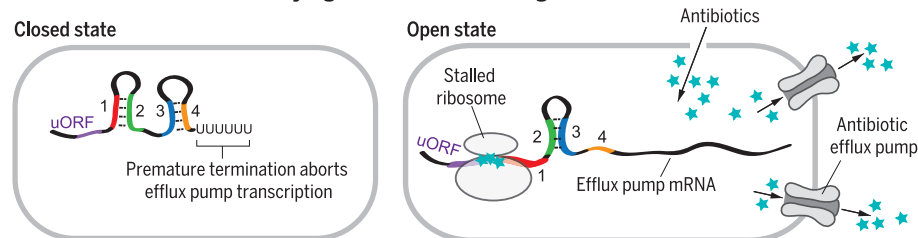
A Genome-wide discovery of riboswitches and attenuators (ribo-regulators) via high precision mapping of RNA 3' ends (term-seq)



Discovery of antibiotic-responsive ribo-regulators in pathogenic and commensal bacteria



B Mechanism of the *L. monocytogenes* *lmo0919* ribo-regulator



Genome-wide discovery of antibiotic-responsive ribo-regulation in bacteria. (A) Direct mapping of RNA 3' ends reveals gene regulation by conditional termination in a genome-wide manner. Differential sequencing of monoculture or complex bacterial communities under metabolite-rich and -poor conditions (metabolite in this case being an antibiotic) detects ribo-regulators that specifically respond to the metabolite. (B) Mechanism of regulation of the *Listeria monocytogenes* *lmo0919* antibiotic responsive ribo-regulator.

RESEARCH ARTICLE

TRANSCRIPTION

Term-seq reveals abundant ribo-regulation of antibiotics resistance in bacteria

Daniel Dar,¹ Maya Shamir,¹ J. R. Mellin,^{2,3,4} Mikael Koutero,^{2,3,4}
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Riboswitches and attenuators are cis-regulatory RNA elements, most of which control bacterial gene expression via metabolite-mediated, premature transcription termination. We developed an unbiased experimental approach for genome-wide discovery of such ribo-regulators in bacteria. We also devised an experimental platform that quantitatively measures the in vivo activity of all such regulators in parallel and enables rapid screening for ribo-regulators that respond to metabolites of choice. Using this approach, we detected numerous antibiotic-responsive ribo-regulators that control antibiotic resistance genes in pathogens and in the human microbiome. Studying one such regulator in *Listeria monocytogenes* revealed an attenuation mechanism mediated by antibiotic-stalled ribosomes. Our results expose broad roles for conditional termination in regulating antibiotic resistance and provide a tool for discovering riboswitches and attenuators that respond to previously unknown ligands.

Riboswitches and attenuators are 5' untranslated region (5'UTR)-residing, cis-regulatory RNA elements (ribo-regulators) that tune gene expression in bacteria by sensing key metabolites, amino acids, nucleotides, and ions (1–6). These RNA elements can regulate the expression of the downstream gene either at the transcription or the translation level. When riboswitches and attenuators control transcription, they usually generate a condition-specific, regulated transcriptional terminator so that termination results in a prematurely aborted transcript, whereas read-through generates a full-length, productive mRNA (Fig. 1A) (5). In the case of riboswitches, the 5'UTR RNA sensor differentially folds to form a terminator or an antiterminator in the presence or absence of a regulating metabolite, respectively; in attenuators, the formation of a transcriptional terminator is mediated by the rate of translation of an upstream open reading frame (uORF), as exemplified in the Trp operon (4). Regulation by conditional termination controls key processes in bacteria, including core metabolism (7, 8), motility (9), biofilm formation (9, 10), and virulence (11, 12). Riboswitches enable optimization of metabolite production in bacterial expression systems (13, 14), are readily applicable components for synthetic biology applications (15, 16), and also form potential therapeutic targets for new antibiotics (17, 18).

Only ~25 classes of naturally occurring riboswitches have been described to date, although it is estimated that hundreds more exist in bacteria (19). Almost all known riboswitches have been discovered via comparative genomics-based approaches comparing intergenic regions across bacterial phyla (20). Once a conserved 5'UTR is detected, its possible ligand is predicted on the basis of the identity of the downstream gene, and these predictions are then subject to extensive in vitro verification. This approach has identified riboswitches conserved across a wide phylogenetic range (19). However, most of the yet-to-be-discovered elements are predicted to be restricted to specific clades of bacteria, and for such elements, current conservation-based approaches are not appropriate (19). We currently lack an experimental method that enables genome-wide discovery of riboswitches and conditional termination regulators. Furthermore, given a metabolite of interest, there is no efficient approach that can identify natural riboswitches or attenuators that sense and respond to it.

Genome-wide mapping of RNA 3' termini via term-seq

We developed an unbiased experimental approach for genome-wide discovery of conditional, regulated transcriptional termination in bacteria. For this, we first mapped all RNA termini that are present in the cell at a given condition. As opposed to eukaryotic RNA, in which the presence of the nearly universal polyadenylate tail allows direct reverse transcription priming from the 3' end, the absence of 3' polyadenylation in bacterial mRNAs makes 3' ends-mapping challenging (21, 22). We developed a RNA-sequencing

(RNA-seq) protocol (denoted here “term-seq”) that directly sequences exposed RNA 3' ends in bacteria, yielding a genome-wide map of RNA termini (Fig. 1B) (23). Applying term-seq to a large set of synthetic transcripts mixed in various, predetermined concentrations verified that term-seq accurately reconstitutes the exact 3' end termini in a highly quantitative manner (fig. S1).

We applied term-seq on *Bacillus subtilis* grown in rich media. The sequencing reads resulting from the term-seq protocol showed >50-fold enrichment for mapping to intergenic regions, implying that sites mapped via term-seq represent native RNA termini existing in the cell (Fig. 1, C and D) (23). Associating each gene with its respective term-seq-inferred termination position in the downstream intergenic region led to identification of 1443 transcript termini (Fig. 1E and table S1), the vast majority of which conformed to the sequence and structural features of rho-independent transcriptional terminators (Fig. 1, F and G, and fig. S2) (23). These results demonstrate the ability of term-seq to map RNA termini to the single-base resolution across the bacterial genome.

A platform for the discovery of genes regulated by conditional termination

We next sought to examine whether term-seq can be used to identify regulated, premature termination events. Genes that are transcriptionally regulated by riboswitches and attenuators will present a premature termination site within their 5'UTR, downstream to the transcription start site (TSS). We therefore also mapped TSSs across the *B. subtilis* genome using a genome-wide 5' end sequencing protocol (23–25) and included standard RNA-seq coverage data so as to gain a comprehensive view of the *B. subtilis* transcriptome (Fig. 1E) (23). Examining known *B. subtilis* riboswitches showed reproducible premature termination sites at the 5' UTR, supporting that such sites can be indicative of riboswitch activity (Fig. 2, A and B).

To assess the sensitivity of this method in revealing genes controlled by regulated termination, we searched for all genes that contained a reproducible term-seq site within their 5'UTRs (23). This search recovered 49 (92%) of the 53 transcriptional units (TUs) regulated by known riboswitches in the *B. subtilis* genome (Fig. 2C and table S2) (23). Four known riboswitch-regulated genes escaped detection, either because they were under the control of multiple consecutive riboswitches, lacked a mapped TSS, or because of an annotation error that placed the riboswitch within a misannotated ORF (fig. S3). These results therefore show a high sensitivity for our method in mapping riboswitches in a genome-wide manner.

Overall, our search retrieved 82 candidate regulatory elements, of which 64 (78%) were mapped to previously reported elements (Fig. 2C and table S2). In addition to the 49 known riboswitches, we also recovered 11 cases of conditional termination known to be regulated by RNA-binding antitermination proteins, including TRAP (26), GlpP (27), PyrR (28), and PTS system proteins (29). We also identified one case of known attenuation

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(30), as well as three elements (the *rimP*, *Pan*, and *vmlR* leaders) predicted as cis-regulatory elements in *B. subtilis* but for which the mechanism of regulation is unknown (20, 31, 32).

Because *B. subtilis* is a highly studied model organism, it has one of the best annotated genomes in the bacterial domain. Nevertheless, we detected 18 previously unidentified elements predicted to regulate gene expression by premature termination (Fig. 2C and tables S2 and S3). These included predicted regulatory elements upstream of the formate dehydrogenase gene *yrhE* (Fig. 2D); the guanosine 5'-monophosphate (GMP) synthase gene *guaA*; *yfnI*, a gene responsible for the biosynthesis of the polyglycerolphosphate moiety of

lipoteichoic acid; and *yxjB*, a 23S ribosomal RNA (rRNA) (guanine748-N1)-methyltransferase predicted to confer resistance to macrolide antibiotics (Fig. 2E). The various functions encoded by the genes newly associated with these regulators, and the lack of homology to any known riboswitch or other RNA elements in the RNA family database (Rfam) (33), suggest that these regulatory elements may respond to previously unidentified ligands, antitermination proteins, or attenuation principles.

We then applied term-seq on *Listeria monocytogenes*, a foodborne pathogen that causes gastroenteritis and can lead to severe sepsis and meningitis in immunocompromised and elderly

patients and abortions in pregnant women (34), and *Enterococcus faecalis*, a causal agent of community and nosocomial infections, including endocarditis and bacteremia (35). Similar to *B. subtilis*, in both pathogens term-seq detected most of the known riboswitches that function via regulated termination, as well as predicted, previously unidentified regulators (Fig. 2, C and F to I, and tables S4 and S5). In *L. monocytogenes*, many of the elements we detected were previously annotated as small RNAs or as potential cis-acting regulatory 5'UTRs (25, 36); our data supports that they are indeed cis-acting regulators (Fig. 2, F and G, and table S4). The conditional termination-based elements we

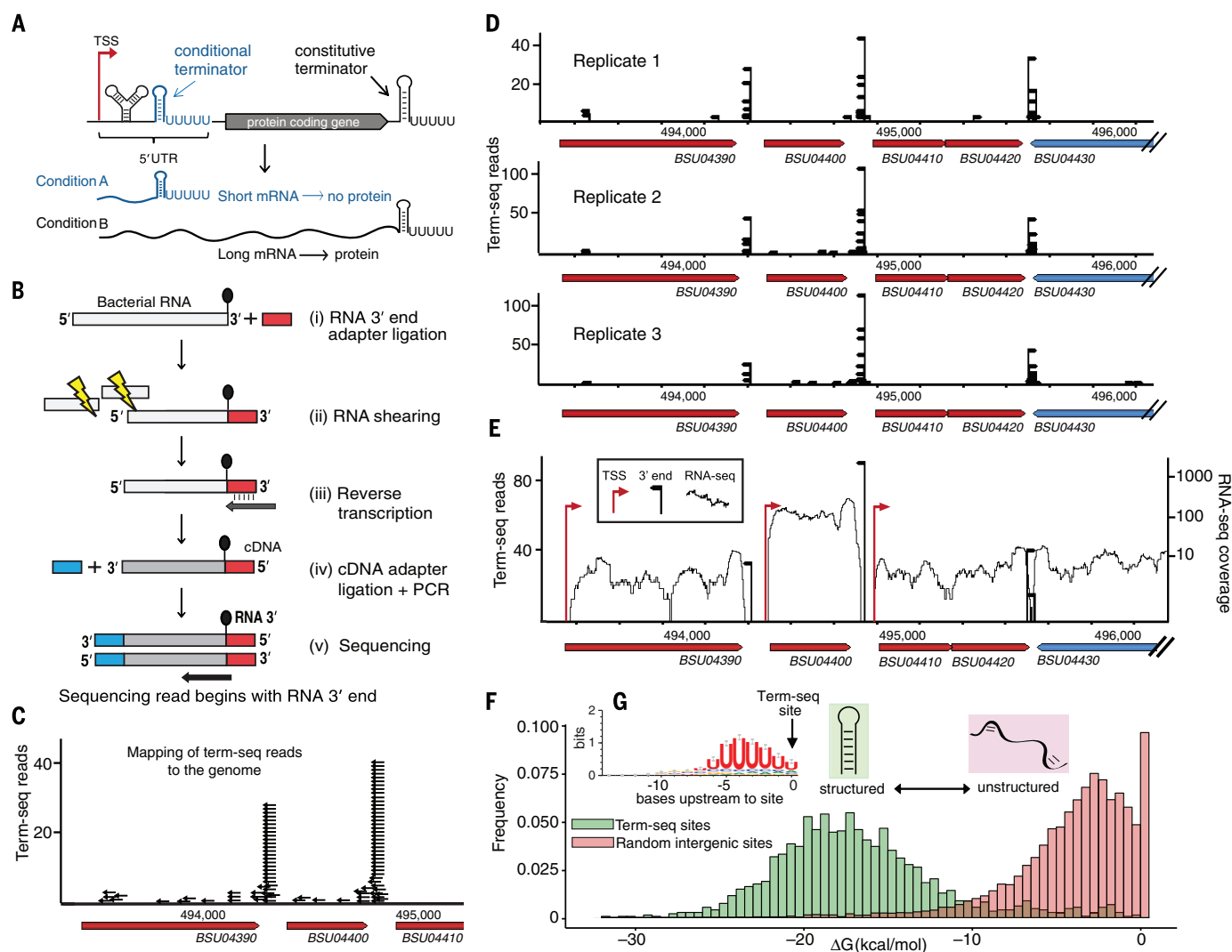


Fig. 1. Term-seq maps RNA termini across the genome. (A) Regulation by conditional termination in bacteria. A model 5' UTR containing a ribo-regulator (riboswitch, protein-binding leader, or attenuator) that differentially folds to generate a condition-specific premature terminator. (B) Schematic representation of the term-seq protocol. (C) Mapping of term-seq reads to the genome yields a typical pattern in which the majority of reads map to discrete intergenic positions marking RNA 3' ends. Black arrows represent individual mapped reads. (D) Data from three biological replicates over a representative 3-kb window of the *B. subtilis* genome show reproducibility. Black arrowheads represent positions supported by term-seq reads, with

arrow height (y axis) representing the number of reads supporting the position. (E) Multilayered RNA sequencing data provides an integrative view of the bacterial transcriptome. Black arrowheads represent predicted term-seq termination sites, with arrow height indicating the average number of reads in three biological replicates. Black curve represents RNA-seq coverage. Red arrowheads mark the position of TSSs, as inferred from transcriptome-wide sequencing of RNA 5' ends (23–25). (F) Folding energy of RNA termini predicted by term-seq ($n = 1443$ sites, green bars) compared with random intergenic sites ($n = 10,000$ sites, red bars). (G) Uridine-rich tail upstream to term-seq sites ($n = 1443$ sites).

detected regulate genes of diverse functions, including some involved in virulence. For example, deletion of the conditional-termination regulator of *lmo0559*, called *rli31* (Fig. 2F), led to an attenuated virulence phenotype of *L. monocytogenes* in mouse and butterfly infection models (37). Deletion of *rli31* also led to decreased lysozyme resistance of *L. monocytogenes* in blood (38). These results imply that the termination-based regulatory elements we detected may control

multiple physiological- and pathogenicity-relevant processes.

Genome-wide metabolite screening in physiological conditions

Although our data uncover multiple cases of new candidate regulators, they do not reveal the metabolites to which these regulators respond. Discovery of the metabolite that controls the activity of a given candidate regulator is a major chal-

lenge in the field and usually involves in vitro structural probing of the regulator in the presence of various candidate metabolites and/or the construction of reporter assays to monitor the activity of the regulator (1–3).

We developed a term-seq-based strategy to evaluate multiple possible metabolites across all candidate regulators simultaneously in physiological, in vivo conditions. We reasoned that the presence of the metabolite should alter the open/closed state

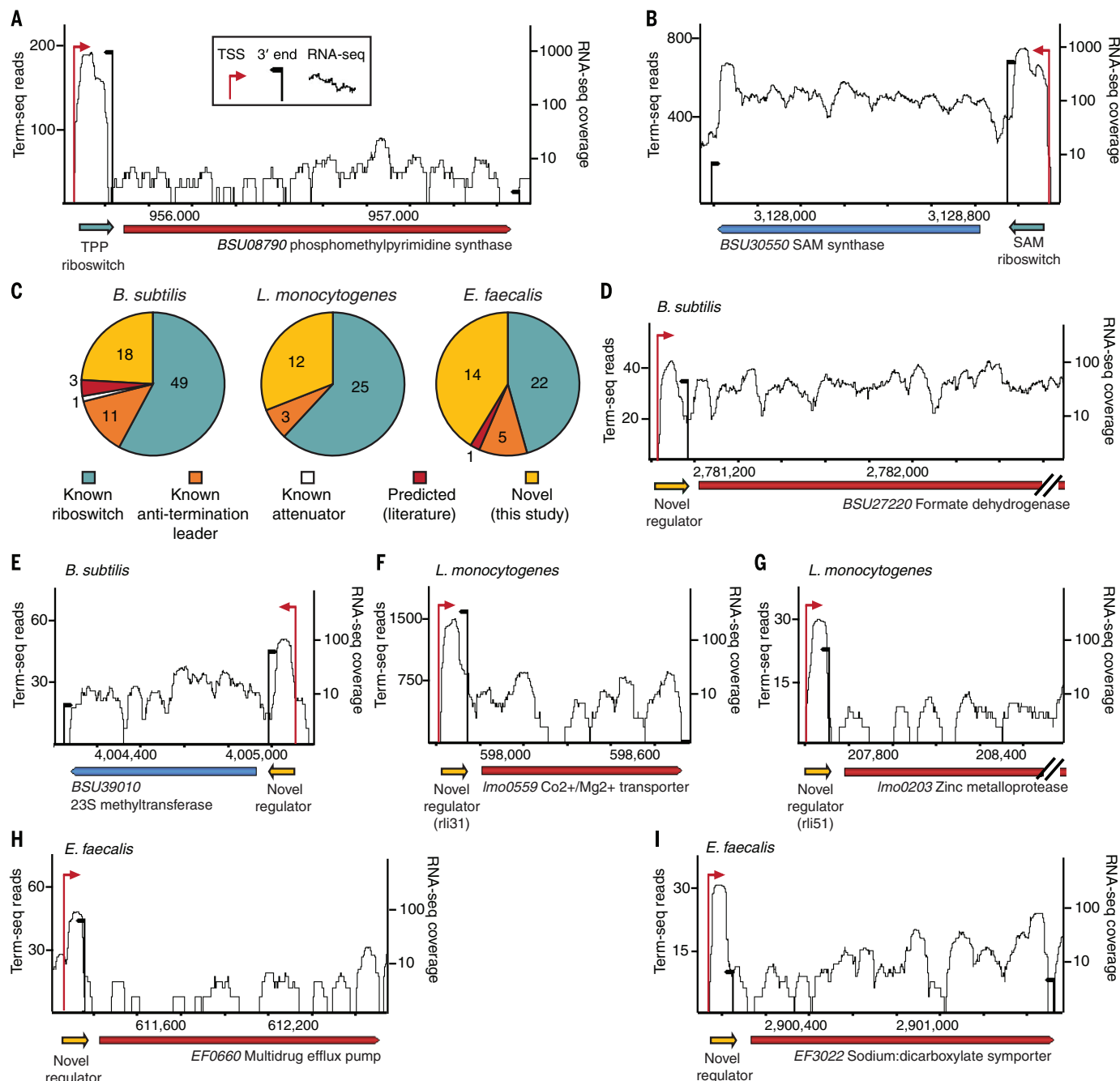


Fig. 2. Discovery of genes regulated by conditional termination. Known riboswitches in *B. subtilis* display a typical pattern of premature termination in the 5'UTR. In both (A) thiamine pyrophosphate (TPP) riboswitch and (B) lysine riboswitch (blue arrows), a term-seq site is observed downstream to the riboswitch. (C) Known and previously unidentified regulators identified by applying term-seq on *B. subtilis*, *L. monocytogenes*, and *E. faecalis*. Pie charts indicate the number of regulators identified in each functional category and organism (tables S2 to S5). (D to I) Examples of previously unidentified regulatory elements (yellow arrows) identified in this study. Axes and colors are as in Fig. 1E.

of the regulator and that this state can be quantitatively measured as the ratio between the full-length RNA and the short, prematurely terminated form (Fig. 3A). Because term-seq directly provides a read-through measure (quantification of short/long transcript ratios) for every expressed regulator in the genome, it enables low-cost, parallel analysis of *in vivo* regulator activities following the application of any metabolite of interest.

To validate this approach, we grew *B. subtilis* in a defined medium with or without the amino acid lysine. Out of the 82 regulators mapped in *B. subtilis*, only the two known lysine riboswitches showed a considerable increase in read-through levels as a result of lysine depletion (Fig. 3, B and C, and fig. S4). Moreover, depletion of a different amino acid from the medium (methionine) (Fig. 3, B and C) did not increase the open/closed ratio of the lysine riboswitches, pointing to high specificity in sensing the presence of lysine.

Antibiotic-controlled conditional termination regulates antibiotic resistance genes

Because inducible antibiotic resistance mechanisms pose a major medical challenge, we screened

for regulators that respond to the presence of antibiotics. The rRNA methylase gene *ermK* confers resistance to macrolide-lincosamide-streptogramin B antibiotics and is controlled by conditional premature termination that is alleviated when the antibiotic is introduced (39). Reports have described similar regulation in additional antibiotics resistance genes, but this mode of regulation was considered rare (30, 32). We thus searched for antibiotic-responsive regulators by applying term-seq to *B. subtilis*, *L. monocytogenes*, and *E. faecalis* after short exposure to sublethal doses of seven different antibiotics (table S6) (23). Six of the regulators we identified (tables S2 to S5) showed an antibiotic-dependent response, characterized by read-through into the downstream gene in the presence of the antibiotic (Fig. 4 and fig. S5).

Two antibiotic-responsive, termination-based regulatory elements were observed in each of the three bacteria studied. In *B. subtilis*, we identified the *vmlR* (32) and *bmrB* (30) regulators, which regulate the expression of antibiotic resistance genes through an unknown or ribosome-mediated mechanism, respectively (Fig. 4, A and B). In *L. monocytogenes*, however, we discovered two

new regulators that were previously annotated as conserved *Listeria* small RNAs (sRNAs) of unknown function, *rli53* and *rli59*, and were hypothesized to act in *cis* (36). We found that these two sRNAs function as antibiotic-responsive riboregulators that control the expression of the genes *lmo0919* and *lmo1652*, both encoding ABC transporter genes of unknown function (Fig. 4, C and D). Whereas the alteration in the open/closed state of *lmo0919* by *rli53* was highly specific to lincomycin, *rli59* was more permissive and responded to several different translation-inhibiting antibiotics (Fig. 4, C and G). In *E. faecalis*, it was previously shown that expression of the *msrC* macrolide-resistance efflux pump increases in response to erythromycin exposure (40). Our data suggest that the *msrC* response results from a coordinated activity of its promoter and a previously unidentified termination-based regulator, which, upon exposure to the antibiotic, act in concert to increase both the transcription initiation rate (7.3-fold) and read-through into the gene (4.6-fold) (Fig. 4, E and G). We detected a similar promoter termination-synchronized activity for the *B. subtilis* *vmlR* gene (Fig. 4B). Last, we found an additional lincomycin-specific

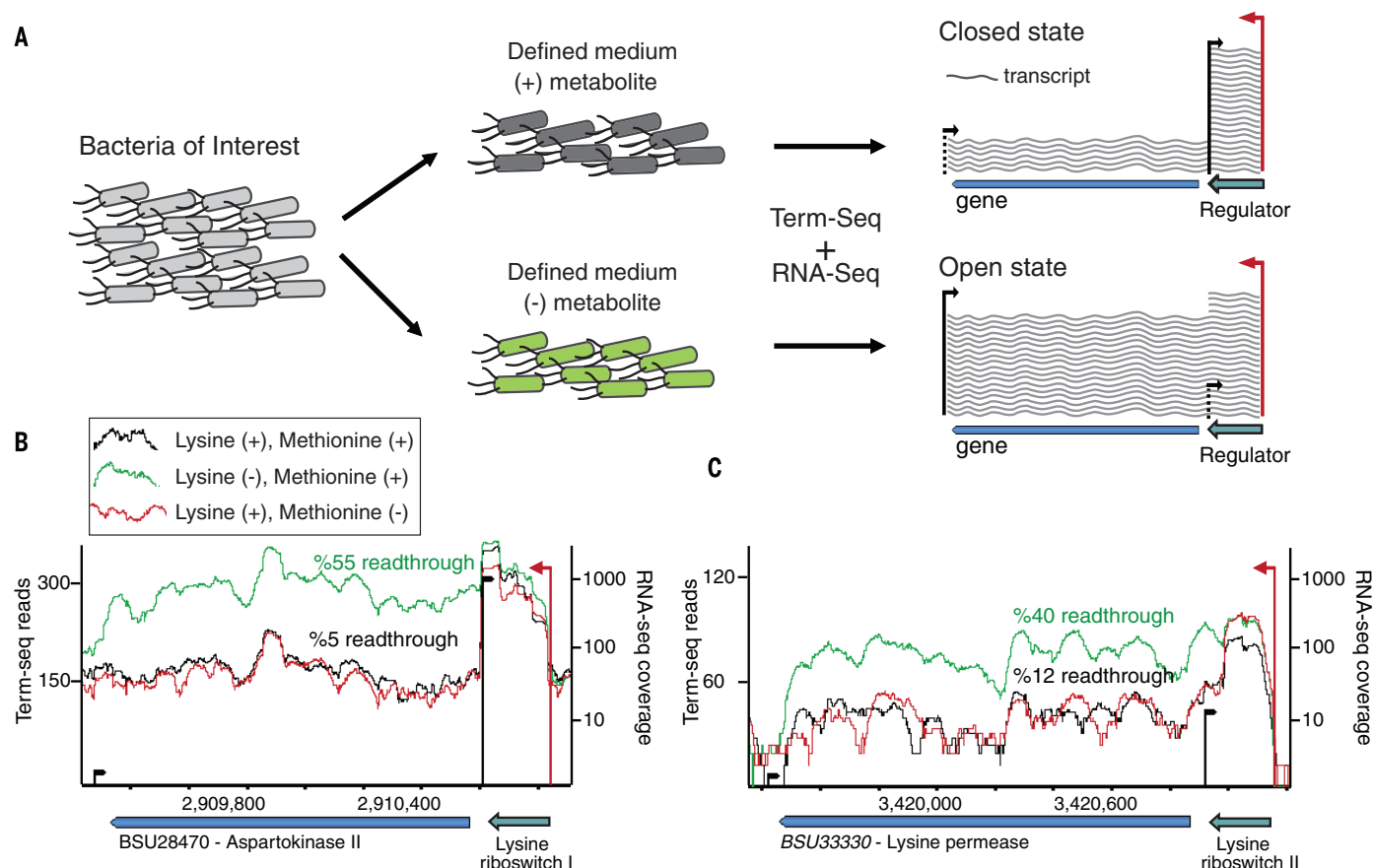


Fig. 3. In vivo metabolite screening by using RNA sequencing. (A) Genome-wide experimental approach for *in vivo* screening of termination-based regulators that respond to a metabolite of choice in physiological conditions. A bacterium of interest is cultured in a defined medium with or without the metabolite of choice. After a brief incubation, RNA is extracted and sequenced by using term-seq and RNA-seq. The long/short transcript ratio, indicative of

the open/closed state of the regulator, can be calculated from term-seq or RNA-seq counts. (B and C) *B. subtilis* was grown in defined, minimal media either containing both lysine and methionine (black RNA-seq coverage), lacking lysine and containing methionine (green), or containing lysine and lacking methionine (red). RNA-seq coverage was normalized by the number of uniquely mapped reads in each sequencing library.

termination-based regulator that controls the expression of yet another ABC transporter of unknown function, *EF2720*, in *E. faecalis* (Fig. 4F).

The presence of antibiotic-responsive, termination-based regulatory elements upstream of specific genes in *L. monocytogenes* and *E. faecalis* suggest that these are possible, previously unknown antibiotic resistance genes. We further characterized the antibiotic-based regulation of *lmo0919*, an ABC transporter of unknown function in *L. monocytogenes*. This gene was previously suggested to be involved

in antibiotic resistance on the basis of its distant homology to the staphylococcal *Vga* gene and its heterologous activity in staphylococcal hosts (41), but its function in *L. monocytogenes* remained unknown. We found that deletion of *lmo0919* rendered *L. monocytogenes* fourfold more sensitive to the antibiotic lincomycin but did not reduce the minimum inhibitory concentration (MIC) of other antibiotic classes (table S7). The protein encoded by *lmo0919* therefore confers lincomycin-specific antibiotic resistance, which is consistent with the

specific activation of its 5'UTR regulator by lincomycin but not by erythromycin or chloramphenicol (Fig. 4, D and G).

The mechanism of antibiotics-mediated conditional termination in *lmo0919*

Inspection of the regulatory 5'UTR sequence of *lmo0919* revealed a potential two-stem, terminator/antiterminator structure (Fig. 5A). Such structures are common in riboswitches and attenuators and can adopt two alternative conformations: one that

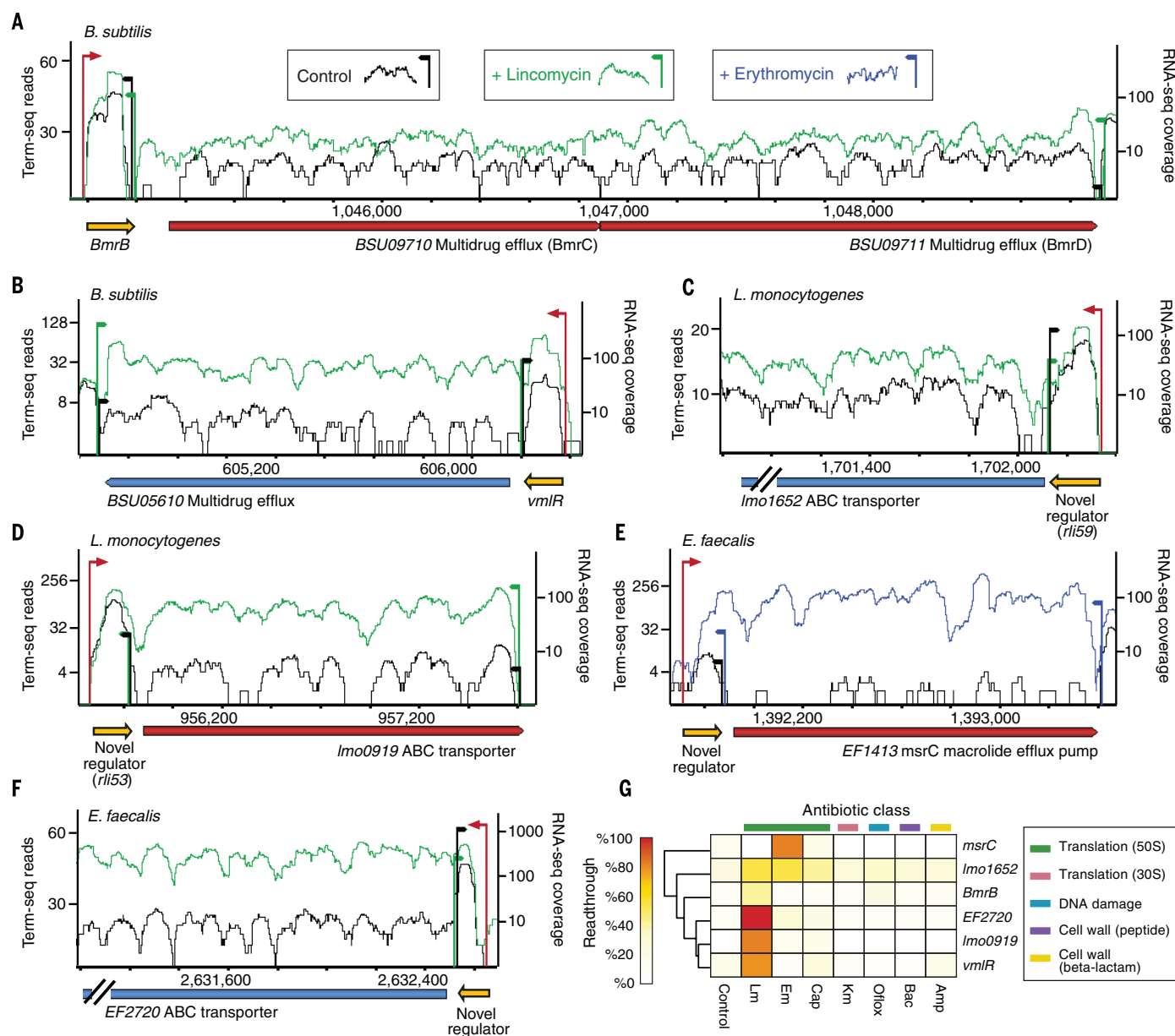


Fig. 4. Antibiotic-responsive conditional terminators. The antibiotic-dependent response of known and previously unidentified regulators as measured in vivo by means of term-seq and RNA-seq. Black, green, and blue RNA-seq coverage and term-seq sites denote the control (LB), lincomycin, and erythromycin conditions, respectively. Term-seq sites represent average read coverage across three biological replicates. (A) The *B. subtilis* *bmrCD* operon. (B) The *B. subtilis* *vmlR* gene. (C to F) Antibiotic dependent tran-

scriptional read-through in previously unidentified regulators discovered in *L. monocytogenes* and *E. faecalis*. (G) Condition-specific read-through calculated in the control and the seven antibiotics exposure experiments. The antibiotic class is defined by the cellular process/component targeted. RNA-seq was normalized as in Fig. 3. Lm, lincomycin; Em, erythromycin; Cap, chloramphenicol; Km, kanamycin; Oflox, ofloxacin; Bac, bacitracin; and Amp, ampicillin.

generates a transcriptional terminator (Fig. 5A, left), and another in which the antiterminator promotes transcriptional read-through by inter-

fering with terminator formation (Fig. 5A, right). To enquire whether this mode of regulation occurs in the case of *lmo0919*, we engineered

mutations in either the first or the second arm of the first stem, disrupting the putative anti-antiterminator or the antiterminator, respectively

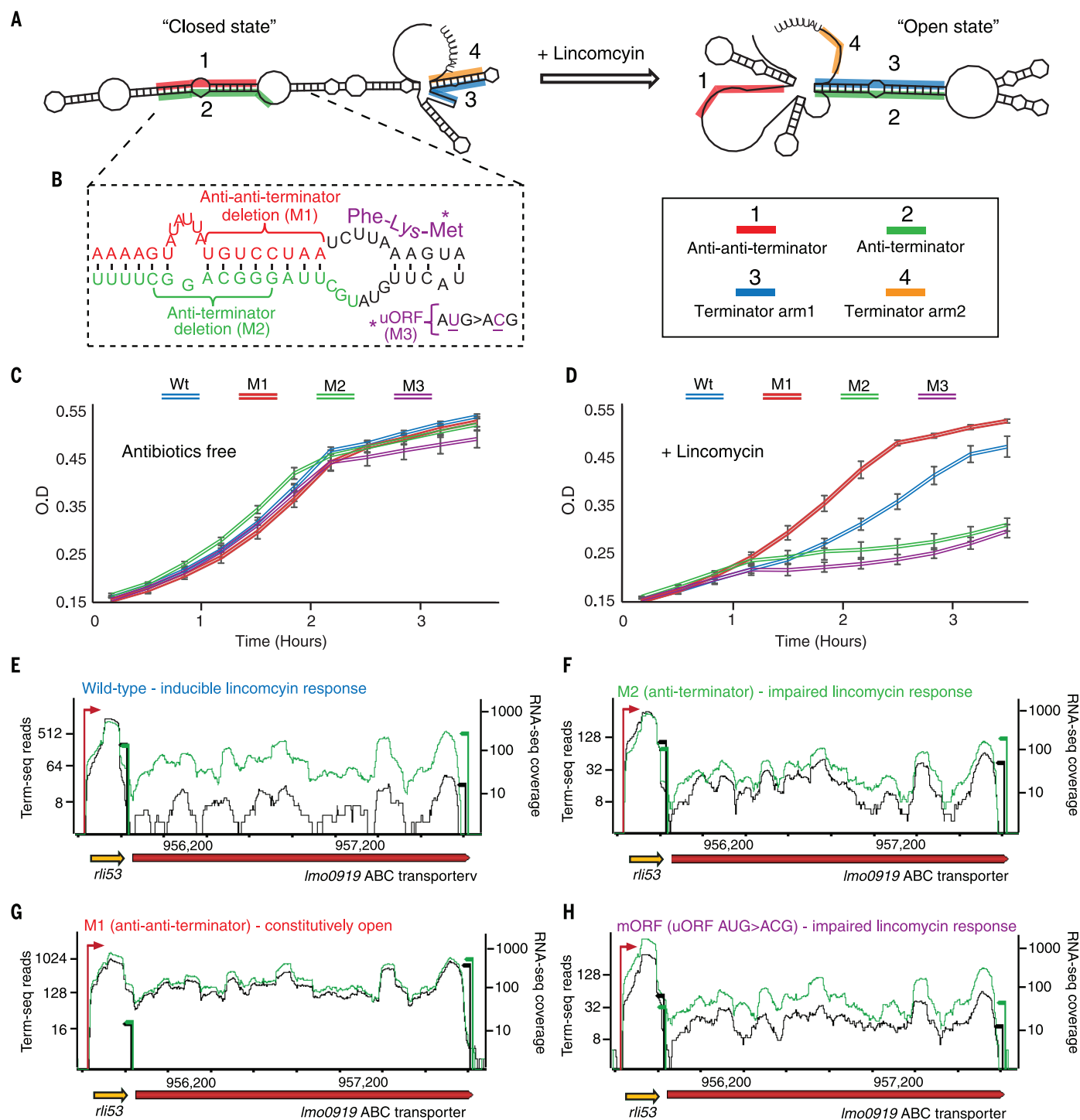


Fig. 5. Antibiotic-responsive terminator/antiterminator RNA structures control the expression of *lmo0919*. Mutational analysis of the 5'UTR of *lmo0919* provides insights into the mechanism of inducible antibiotic resistance. (A) A predicted RNA secondary structure of the *lmo0919* 5'UTR. This element is predicted to form two alternative, mutually exclusive structures that mediate either termination or antibiotic-dependent read-through. (Left) The "closed-state" structure encodes a terminator and an upstream stem. (Right) The "open-state" structure in which the terminator structure is sequestered by an

antiterminator. (B) Generation of mutants that interrupt the anti-antiterminator (red), the antiterminator (green), or a conserved uORF that overlaps the anti-antiterminator (purple). (C and D). Mutants were grown in BHI media without lincomycin (C) or containing 0.5 μ g/ml lincomycin (D), respectively. Error bars represent standard error. (E to H) Term-seq and RNA-seq coverage of wild-type and mutants grown in BHI without lincomycin (black RNA-seq curves and black term-seq sites) or with 0.5 μ g/ml lincomycin (green RNA-seq curves and green term-seq sites). RNA-seq coverage was normalized as in Fig. 3.

(Fig. 5B). Consistent with the model, deletion of eight nucleotides from the antiterminator kept the regulator in a constitutively “closed” state, even in the presence of lincomycin antibiotic, rendering the bacteria sensitive (Fig. 5, B to E and F, and table S8). In contrast, deletion of eight nucleotides from the anti-antiterminator freed the antiterminator to interfere with the terminator structure, leading to constitutive read-through (“open” state), even in the absence of antibiotics, and resulting in increased resistance to lincomycin (Fig. 5, B to E and G). These results support a model in which the lincomycin-dependent activation of *lmo0919* expression is mediated by a structural interplay

of terminator/antiterminator structures in the 5'UTR of this gene.

The structural alterations in the *lmo0919* ribo-regulator could either be mediated by direct binding of the antibiotic to the ribo-regulator (a riboswitch), or by attenuation, in which the lincomycin-inhibited ribosomes stall on an uORF in the ribo-regulator, thus shifting the ribo-regulator structure from a “closed” to an “open” state (as suggested for the *bmrB* regulator in *B. subtilis*) (30). To differentiate between these mechanisms, we measured the lincomycin-dependent induction of *lmo0919* in *L. monocytogenes* expressing the ErmC 23S rRNA methyltransferase (23, 42). In these bacteria, the ribosomes are dimethylated

at position A2058 of the 23S rRNA, rendering the ribosomes resistant to lincomycin (42). In ErmC-expressing bacteria, the *lmo0919* regulator was no longer responsive to lincomycin (fig. S6), suggesting that this ribo-regulator depends on stalled ribosomes for its activity and does not interact directly with the antibiotic molecule.

We then performed a comparative analysis of the 5'UTR sequences of *lmo0919* homologs in various Gram-positive bacteria (23). Although the nucleotide sequence of the ribo-regulator showed almost no conservation between species, the terminator/antiterminator architecture was strictly conserved among all homologs (figs. S7 and S8). Despite the near lack of sequence conservation, all

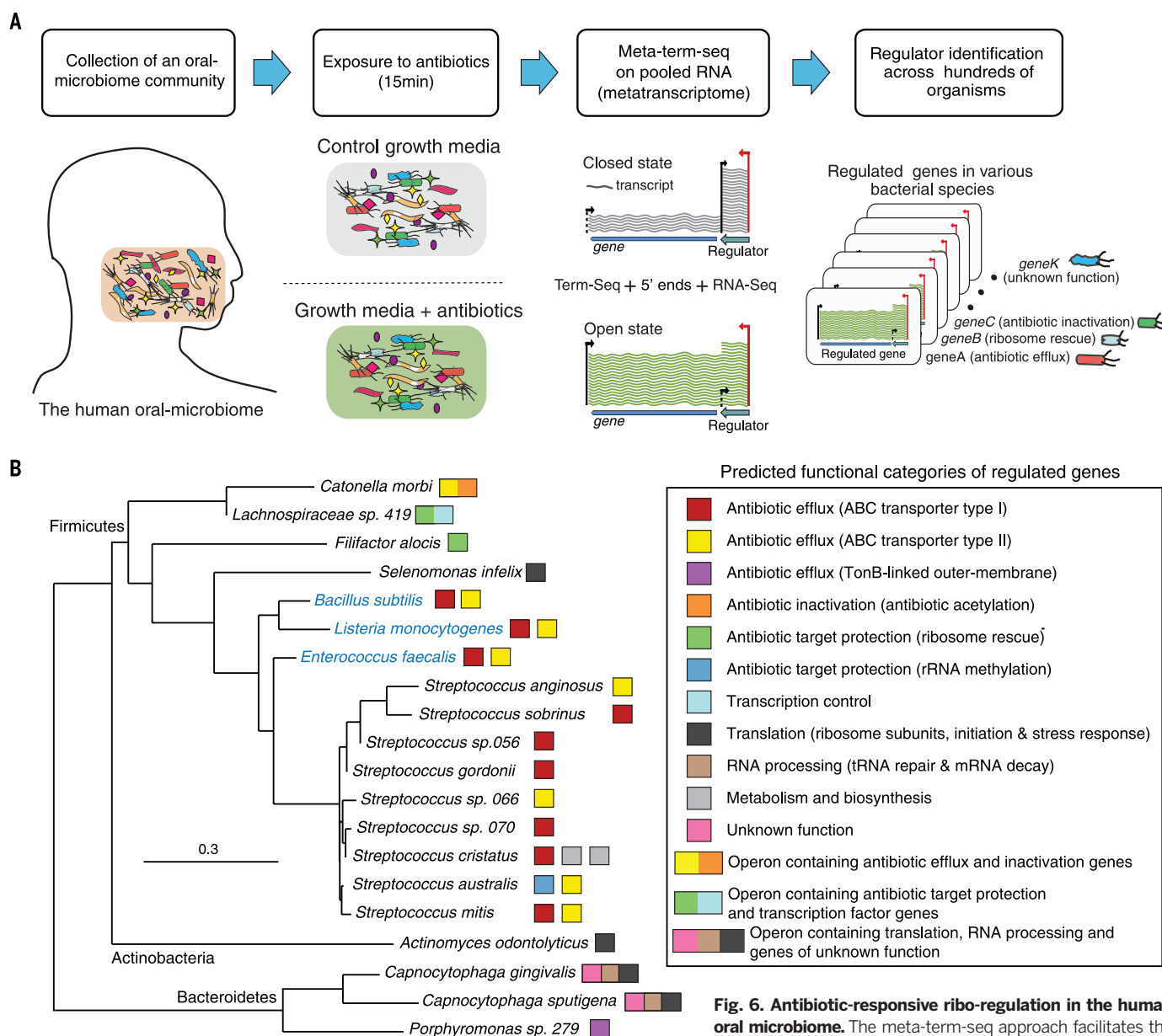


Fig. 6. Antibiotic-responsive ribo-regulation in the human oral microbiome. The meta-term-seq approach facilitates the discovery of metabolite-responsive regulators across complex

bacterial communities. **(A)** Schematics of the meta-term-seq workflow, from sample collection to regulator identification. **(B)** A 16S rRNA phylogenetic tree comprising oral microbiome bacteria found to have one or more lincomycin-responsive regulators (23). The predicted functions of the regulated genes in each species are indicated with colored boxes according to the inset legend. In some cases, a single operon contained several different functions (multicolored rectangles, legend bottom). Individual bacteria studied in monoculture were added to the tree (marked with blue-colored names).

regulators contained a three-amino-acid uORF exactly overlapping the inhibitory anti-antiterminator sequence (Fig. 5B and figs. S7 and S8). Although such small ORFs were never previously reported to be involved in transcriptional attenuation, the strong positional conservation of the uORF in the ribo-regulator led to the hypothesis that its translation forms the basis for the attenuation-based regulation. Indeed, a green fluorescent protein (GFP) fusion assay showed that this uORF is translated in *L. monocytogenes* in vivo (fig. S9) (23).

To characterize the effects of lincomycin on the interaction between the ribo-regulator and the ribosome, we measured the levels of ribosome occupancy over the ribo-regulator in control and lincomycin-treated bacteria using ribosome profiling (Ribo-seq) (23, 43). We detected approximately five- and twofold increases in ribosome occupancy over the three-amino-acid uORFs in *L. monocytogenes* and *Listeria innocua*, respectively, after brief exposure to lincomycin (fig. S10) (23). These results show that lincomycin-inhibited ribosomes specifically stall at the three-amino-acid uORF that overlaps the anti-antiterminator sequence.

Collectively, these results point to an attenuation-based regulatory mechanism in which the association of the ribosome with the antibiotic leads the ribosome to stall on a nine-base uORF that overlaps the anti-antiterminator, releasing the antiterminator to interfere with terminator folding and thus allowing read-through into the antibiotic resistance gene. Indeed, a single-base mutation that changed the ATG (Met) initiation codon of the uORF into ACG led to suppressed lincomycin-dependent read-through, validating the model (Fig. 5, B, D, and H).

The *lmo0919* regulator is specifically activated by lincomycin but not by erythromycin (Fig. 4), although both antibiotics induce ribosome stalling. Although antibiotics of the lincomycin family inhibit ribosome progression after the incorporation of one to two amino acids, erythromycin requires the addition of six to eight amino acids to the nascent chain before it stalls the ribosome (44). It is therefore possible that the specificity of the *lmo0919* ribo-regulator to lincomycin stems from the short size of its functional uORF.

Abundance of antibiotic-dependent ribo-regulation in the human microbiome

The presence of multiple antibiotic-responsive, termination-based regulatory elements in three evolutionary distant bacterial species prompted us to hypothesize that this mode of regulation is commonly controlling antibiotic resistance in nature. We thus probed for such regulatory elements in the human oral microbiome, a complex microbial community comprising hundreds of commensal teeth- and mouth-associated bacterial species that are frequently naturally exposed to antibiotics (45).

We used a meta-transcriptomics approach (denoted here “meta-term-seq”) in order to probe the transcriptional profile of the microbial consortium in a single experiment. For this, teeth-associated bacteria were sampled from three healthy individuals and were pooled in tubes containing brain heart infusion (BHI) medium

with and without the antibiotic lincomycin for 15 min. We applied term-seq and RNA-seq on pooled RNA and mapped RNA reads to the >400 reference genomes from the human oral microbiome project (23, 45, 46). We further studied the antibiotic-responsive meta-term-seq profiles in the 167 species that showed considerable expression of at least 10% of their genes (23).

Operons activated by alleviation of premature termination in response to lincomycin were abundantly found in members of the human oral microbiome. We detected 21 regulatory elements, overall controlling 57 genes, in which transcriptional read-through was substantially increased after the application of lincomycin (Fig. 6, fig. S11, and tables S1 and S8) (23). Such elements were detected in 21% (13 of 61) of the Firmicutes studied, indicating that this mode of regulation is common in bacteria in this phylum. The genes regulated by the antibiotic-responsive cis-acting RNA elements included several different classes of multidrug antibiotics exporters and efflux pumps (47, 48), rRNA methylases known to confer antibiotic resistance via modification of the ribosomal RNA (39), acetyltransferases known to directly deactivate the antibiotic via acetylation (49), genes annotated as tetracycline resistance small-guanosine triphosphatases that rescue antibiotic-bound ribosomes (50), and additional genes that have no described antibiotic resistance (Fig. 6, fig. S11, and tables S1 and S8). These results highlight meta-term-seq as a method with which to probe gene regulation in microbial consortia and reveal a common control mechanism for antibiotic-resistance genes in human-associated bacteria.

Discussion

We present here an unbiased experimental method for high-throughput discovery of conditional termination-based regulators in individual bacteria as well as in microbiomes. Moreover, we developed a screening procedure that measures the in vivo read-through levels of every regulator in the genome in parallel, thus enabling the identification of regulators that specifically respond to a given metabolite. By screening for ribo-regulators that respond to antibiotic molecules, we highlight a common form of regulation of antibiotics resistance genes and expose the molecular details entailing its mechanism of action.

Through the use of term-seq, which does not rely on comparative genomics, we overcome the challenge of identifying highly divergent or evolutionarily new regulators, as well as short regulators, both of which are generally challenging or even impossible to find when relying on sequence conservation (19). Furthermore, with the ability of term-seq to measure the in vivo activity of every expressed regulator in the cell in parallel, we prevent experimental biases that stem from artificial transfer of the regulator to a model organism and allow for large-scale screens and studies of regulators in organisms that lack genetic engineering tools. In addition, our meta-term-seq approach enables parallel discovery of ribo-regulators in multiple organisms belonging to a bacterial consortium, even if these organisms

are noncultivated. Nevertheless, although our approach identifies metabolite-responsive regulators, it does not differentiate between ribo-switches, attenuators, and protein-dependent termination and moreover cannot identify ribo-switches and attenuators functioning via translation inhibition rather than premature termination. Our approach will also work only for metabolites that can enter the cell (for direct binding to ribo-switches) or can be sensed by the cell. Last, another limitation of the approach is that the read-through response we record in the regulator may be due to a metabolite different than the one added in the screen. This would occur, for example, when the added metabolite leads to changes in the levels of the true metabolite. However, because the riboswitch-mediated response is direct and hence expected to be very rapid, these secondary effects could be mitigated if the exposure to the screened-for metabolite is very short, as done in our antibiotic-exposure experiments.

Termination-based regulation of antibiotic resistance genes have so far been sporadically described (30, 32, 39). Our identification of numerous regulators in model organisms and in many species of the human oral microbiome indicates that such regulation of antibiotic resistance genes is very common in Gram-positive bacteria. Moreover, even in the three model organisms that we studied in depth, additional antibiotic-dependent regulators may be present. For example, in *B. subtilis* we identified a previously unknown regulator upstream to *BSU39010* (Fig. 2E). In *Streptomyces fradiae*, this gene methylates the 23S rRNA at position G748, which was shown to provide a highly specific resistance to the macrolide antibiotic tylosin, but not to other macrolides (51). Although we did not observe an antibiotic-dependent response in this regulator, it is conceivable that it would show a specific response to tylosin, which was not included in our screen. In addition, regulators were found for the *L. monocytogenes* *lmo2760* and the *E. faecalis* *EF0660* genes (Fig. 2H), both of which are homologous to multidrug efflux pumps. These regulators possibly respond to antibiotics other than the ones we used in our screen.

The importance of termination-based cis-regulators in maintaining the versatile physiology of bacteria is evident from their nearly ubiquitous distribution across the bacterial kingdom (33). We now provide the tools with which we can identify and characterize many of these in the future.

Materials and methods

Oligonucleotides, wild-type bacterial strains, and culture conditions

All oligonucleotides used in this study were purchased from Sigma (St. Louis, Missouri) or Integrated DNA Technologies (IDT, San Jose, California) (table S9). *Bacillus subtilis* str. 168, *Listeria monocytogenes* EGDe, *Listeria innocua* Clip11262, and *Enterococcus faecalis* ATCC 29212 were cultured under aerobic conditions at 37°C with shaking in either LB (10 g/L tryptone, 5 g/L yeast extract 5 g/L NaCl), TB (12 g/L tryptone, 24 g/L yeast extract, 0.4% glycerol, 2.2 g/L KH_2PO_4 and 9.4 g/L K_2HPO_4),

BHI broth (Difco, Franklin Lakes, New Jersey), or M9 minimal media [0.5% w/v glucose, 2 g/L $[\text{NH}_4]_2\text{SO}_4$, 18.3 g/L $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 6 g/L KH_2PO_4 , 1 g/L sodium citrate, 0.2 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 μM MnCl_2 , and 5 μM CaCl_2 , tryptophan (Sigma) 50 $\mu\text{g}/\text{mL}$].

Lysine-responsive regulation

B. subtilis was grown overnight in LB and then diluted 1:200 into 150 ml of M9 media supplemented with lysine and methionine (50 $\mu\text{g}/\text{mL}$ each). Bacteria were grown to $\text{OD}_{600} = 0.9$ to 1.0, washed, and then resuspended to an $\text{OD}_{600} = 0.3$ in 3 ml of M9 media, containing the following combinations of amino acids at a final concentration of 50 $\mu\text{g}/\text{mL}$: lysine and methionine ($\text{lys}^+ \text{met}^+$), methionine only ($\text{lys}^- \text{met}^+$), or lysine only ($\text{lys}^+ \text{met}^-$). Cells were incubated for 2 hours and collected by means of centrifugation (4000 rpm, 5 min, and 4°C) followed by flash freezing. Samples were stored in -80°C until RNA extraction.

Antibiotics-responsive regulators

A sublethal concentration for the antibiotics lincomycin, erythromycin, chloramphenicol, kanamycin, ofloxacin, ampicillin, and bacitracin (Sigma) was determined for each of the organisms used in this study as follows. Bacterial cultures were propagated in LB or BHI overnight in triplicates and diluted 1:200 into fresh media. Cultures were grown to early exponential phase ($\text{OD}_{600} = 0.1$ to 0.2) and then supplemented with serially diluted antibiotics stocks. The growth rate was dynamically monitored in intervals of 10 to 15 min by using a 96-well plate format optical density (OD) reader (Infinite M200 Tecan) for a period of at least 4 hours. The highest antibiotics concentration that did not cause growth-rate inhibition as compared with the no-antibiotics control was chosen as the sublethal dosage (table S7). To identify antibiotic-responsive regulators, bacteria were grown in LB or BHI in triplicates as described above and, upon reaching early exponential phase, 5-ml cultures were independently exposed for 15 min to the sublethal concentration of each antibiotic as determined above. Bacteria were then collected by means of centrifugation, flash frozen, and stored in -80°C until RNA extraction.

RNA isolation

Frozen bacterial pellets were lysed by using the Fastprep homogenizer (MP Biomedicals, Santa Ana, California), and RNA was extracted with the FastRNA PRO blue kit (MP Biomedicals, II6025050) according to the manufacturer's instructions. RNA levels and integrity were determined with Qubit RNA BR Assay Kit (Life technologies, Carlsbad, California, Q10210) and TapeStation (Agilent, Santa Clara, California, 5067-5576), respectively. All RNA samples were treated with TURBO deoxyribonuclease (DNase) (Life technologies, AM2238).

Construction of mutated *Listeria* strains

For mutant generation with pMAD-based plasmids (52), ~600 nucleotide (nt) regions of complementarity both upstream and downstream of a targeted region were either ordered as gBlocks (IDT) and amplified with gBlock-Up and -Down oligo-

nucleotides (table S9) complementary to uniform flanks on each gBlock corresponding to the 40 nt on either side of the pMAD multicloning site, or polymerase chain reaction (PCR) amplified with Phusion High fidelity polymerase and reagents (Finnzymes, F-553) by using genomic DNA as a template and then joined by a second splice overlap extension PCR reaction by using the first two PCR products as template to generate an Upstream-Downstream (UD) PCR product (table S9, *lmo0919* deletion). PCR products were subsequently purified with QIAquick PCR purification columns (Qiagen, Hilden, Germany, 28104), digested with the Sall and XmaI restriction enzymes [New England BioLabs (NEB), Ipswich, Massachusetts], purified again as before, and ligated into Sall/XmaI digested pMAD plasmid for 1 hour at 25°C with T4 DNA ligase (NEB, M0202S). Two microliters of each ligation were transformed into chemically competent *Escherichia coli* Top10 (Invitrogen, Carlsbad, California, C404003) cells according to the manufacturer's instructions. Transformants were screened by means of PCR and Sanger sequencing for the presence of the appropriate insert. Electrocompetent *L. monocytogenes* strains were transformed with the respective plasmid and mutagenesis carried out as described previously (52). Briefly, after transformation and plating onto selective BHI, 5 $\mu\text{g}/\text{mL}$ erythromycin (Em) 80 $\mu\text{g}/\text{mL}$ 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) plates, bacteria were grown at 30°C for 2 days. A single blue colony was picked and transferred to liquid BHI broth and grown for an additional 6 hours at 30°C . The colony was then diluted 1:1000 into 10 ml of BHI-Em and grown overnight at 42°C , which prevents pMAD replication in the cytosol owing to a temperature-sensitive ori. Serial dilutions were plated onto BHI-X-gal-Em plates and grown for 2 days at 42°C , and the process was reiterated several times until white colonies, in which the plasmid integrated into the genome, were recovered. Colonies were screened by means of colony PCR, and mutants were confirmed with Sanger sequencing.

For the construction of the GFP reporter strain, a translational fusion was constructed by generating a synthetic 1936-base pair (bp) fragment (*rli53*-GFP) encompassing the upstream region of *rli53* (591 bp), the 5' of *rli53* (40 bp) and a small spacer (30 nt) fused to the GFP optimized for *Listeria* (53) that lacks the initiator codon and followed by the downstream region of *rli53* (549 bp) (IDT) (table S9). The construction was cloned into the BamHI and EcoRI restriction sites of the suicide vector pMAD (52) in order to generate the plasmid pMAD-*rli53*-GFP used to transform the wild-type strain *L. monocytogenes* EGD-e as described above, and generating the *L. monocytogenes* strain expressing the chromosomal fusion *rli53*-GFP. The sequence was verified with DNA sequencing by using primer oligonucleotides (*lmo0918*-upstream and *lmo0919*-downstream) (table S9).

MIC determination

The MIC was determined in broth culturing conditions in the presence of serially diluted anti-

biotic concentrations. Briefly, the bacterial strains were grown overnight at 37°C in BHI agarose plates, and 1 to 3 single colonies were collected into 1 ml of BHI broth. The OD_{600} was adjusted to 0.01 and then diluted 1:10 into a 96-well plate containing a final volume of 200 μL BHI supplemented with twofold serial dilutions of lincomycin, erythromycin, and chloramphenicol. The samples were grown over 2 days at 37°C without shaking, and the MIC was determined as the lowest antibiotic concentration to fully inhibit growth.

Term-seq library preparation

DNase-treated RNA (1 to 5 μg) was subjected to a 3' end-specific ligation by mixing 5 μL of RNA solution with 1 μL of a 150 μM DNA adapter solution (table S9), 2.5 μL of 10X T4 RNA ligaseI buffer, 2.5 μL of 10 mM adenosine triphosphate, 2 μL of dimethyl sulfoxide, 9.5 μL 50% PEG8000, and 2.5 μL of T4 RNA ligaseI enzyme (NEB, M0204). The reaction was incubated for 2.5 hours at 23°C and then cleaned by adding $2.2\times$ (55 μL) paramagnetic SPRI beads (Agencourt AMPure XP, Beckman Coulter, Brea, California), mixing well by pipetting and leaving the reaction-bead solution to rest at room temperature for 2 min. The supernatant was separated from the beads by using a 96-well magnetic separator (Invitrogen). Beads were washed on magnet (beads securely attached) by discarding the solution and adding 120 μL of 70% ethanol (EtOH), allowing an incubation period of 1 min. The cleanup stage was repeated, and the beads were air dried for 5 min. The RNA was eluted in 5 to 10 μL of H_2O . The RNA was fragmented with fragmentation buffer (Ambion) in 72°C for 1.5 min. The fragmentation reaction was cleaned by using SPRI beads $2.2\times$ as described above and eluted in 28 μL of H_2O . Ribosomal RNA was depleted by using the Ribo-Zero rRNA Removal Kit (epicenter, MRZB12424) or MICROBExpress (Life technologies, AM1905) according to the manufacturer's instructions. Depleted RNA was reverse transcribed by incubating 11 μL of RNA with 1 μL of 10 μM reverse transcription primer (table S9), incubating at 65°C for 5 min, and immediately placing on ice for 2 min. Two microliters of AffinityScript reverse transcriptase (Agilent, 600559), 2 μL of 10X Affinity Script Buffer, 2 μL of 100 mM DTT, and 2 μL of 10 mM deoxynucleotide triphosphates (Sigma, D7295) were added, and the reaction was incubated at 42°C for 45 min and then terminated by incubation at 75°C for 15 min. To degrade the RNA template, 1 μL of ribonuclease (RNase) H (NEB, M0297) was added, and the reaction was incubated for an additional 30 min at 37°C . The reaction was cleaned by using SPRI beads at a $2.2\times$ ratio (46 μL) and eluted in 5.5 μL of H_2O . Five microliters of the resulting cDNA was subjected to a second 3' end ligation, as above, but using a cDNA-specific ligation adapter (table S9). The reaction was incubated at 23°C for 4 to 8 hours and then cleaned with SPRI beads at a $1.8\times$ ratio (45 μL), eluting the cDNA in 23 μL of H_2O . Twenty-two microliters of ligated cDNA solution was mixed with 1.5 μL of forward and reverse primers, at 25 μM each (table S9) and 25 μL of KAPA Hi-Fi PCR-ready mix (KAPA Biosystems,

Wilmington, Massachusetts, KK2601). The library was amplified by using the manufacturer's protocol with 16 to 18 amplification cycles. The final term-seq library was cleaned with SPRI beads at a 0.9× ratio (45 µl), and the concentration and size distribution were determined with the Qubit dsDNA BR Assay Kit (Life technologies, Q32850) and the dsDNA D1000 TapeStation kit (Agilent, 5067-5582), respectively.

RNA-seq and 5' end sequencing

For RNA-seq library preparation, 4 µg of DNase-treated RNA was fragmented in 20 µL of reaction volume as described above and cleaned by adding 2.5× (50 µl) SPRI beads and 30% v/v isopropanol (30 µl). The beads were washed with 120 µl of 80% EtOH and then air dried as described above. The RNA was eluted in 26 µl of H₂O, and rRNA was depleted as in term-seq. Strand-specific RNA-seq was performed with the NEBNext Ultra Directional RNA Library Prep Kit (NEB, E7420) with the following adjustments to the manufacturer's instructions: All cleaning steps were carried out with 2.5× SPRI beads and 30% v/v isopropanol combinations, the washing steps were performed with 450 µl of 80% EtOH, and only one cleanup step was performed after the end repair step. The resulting libraries concentrations and sizes were evaluated as in term-seq. For 5' end sequencing, the RNA was divided into a tobacco acid pyrophosphatase (TAP)-treated and untreated (noTAP) reactions, which were subsequently sequenced by using a 5' end-specific library preparation protocol described in (25). In *B. subtilis*, the 5' end libraries were prepared with bacteria grown to early exponential phase in TB medium. For *L. monocytogenes*, 5' end data was taken from (25).

Deep-sequencing, read mapping, and counting

RNA-seq, 5' end, and term-seq libraries generated in this study (table S10) were sequenced by using the Illumina Miseq, HiSeq. 1500, or NextSeq. 500 platforms. Sequenced reads were demultiplexed, and adapters were trimmed by using Casava v1.8.2. Reads were mapped to the reference genomes (Gene annotation and sequences were downloaded from GenBank: AL009126, NC_003210, and NC_003212 NC_004668 for *Bacillus subtilis* str. 168, *Listeria monocytogenes* EGD-e, *Listeria innocua* Clp11262, and *Enterococcus faecalis* V583, respectively) by using NovoAlign (Novocraft) v3.02.02 with default parameters, discarding reads that were nonuniquely mapped as previously described in (25). All downstream analyses were performed by using custom written perl and R scripts (available through GitHub under repository aad9822).

RNA-seq-mapped reads were used to generate genome-wide RNA-seq coverage maps. 5' end and term-seq positions were determined as the first nucleotide position of the mapped read. Total 5' end or term-seq coverage was calculated per nucleotide position in the genome. The data was visualized by using a custom browser as described in (25) (Figs. 1 to 5). TSSs were determined as in

(25). Briefly, the ratio between TAP-treated (TAP) and untreated (noTAP) was calculated for each genomic position covered by least four reads in the TAP condition. The maximal 5'UTR allowed was set to 450 nt, and the TSS was chosen as the site with a TAP/noTAP ratio greater than 2 for *B. subtilis* and greater than 1 for *L. monocytogenes* and *E. faecalis*. In cases in which multiple potential TSSs were available, the site with the highest coverage was chosen as the gene TSS.

Terminator identification and analysis

For the assignment of terminators to genes, the downstream sequence of each gene (up to 150 nt, allowing up to 10 nt invasion to the next gene) was scanned for term-seq sites that were covered by a minimum of four reads in each of the three biological replicates. In case multiple sites were observed, the site with the highest coverage was selected as the terminator. This filtering resulted in 84.4% (±0.7%) of the reads mapping to non-coding positions, representing >50-fold enrichment over what would have been expected by chance based on the coding:noncoding composition of the *B. subtilis* genome ($P = 0$, binomial exact test). For terminator sequence and structure analysis, the 40-nt upstream and 20-nt downstream sequences were collected for each terminator and folded in silico via the RNAfold software by using the standard parameters (54). For 96% (1382 of 1443) of the sites we predicted as terminators, there was a clear stem/loop structure preceding the site, with 91% (1302 of 1443) of them preceded by at least four uridine residues in the eight bases immediately upstream to the termination position, which is in agreement with the known features of Rho-independent terminators (table S2 and fig. S1). Nucleotide logos were generated by using WebLogo (55).

Previously established terminators inferred by experimental assays such as Northern blots or S1 nuclease mapping of the 3' end (56) were collected for *B. subtilis* genes covered by at least 25 RPKM RNA-seq reads (table S11) and then compared with the term-seq-inferred terminator set described in table S1. We were able to detect 43 of 46 (93%) known, high-confidence terminators (table S11). Two of the three terminators that were not detected with term-seq were associated with genes of relatively low expression (76 and 90 reads per million per kilobase), which could explain why they did not appear with a sufficient number of reads in the three biological repeats to be considered by our filtering criteria.

Discovery of premature termination

For the discovery of premature termination, the 5'UTR (the beginning of which was defined by the TSS) of each gene was scanned for term-seq sites that were covered by a minimum of two reads in each of the three biological replicates. Because the average length of a terminator is ~20 to 25 nt (56), only 5'UTRs in which the distance between the TSS and the term-seq site was at least 70 nt were considered. Candidate regulators

that displayed high term-seq density also across the gene body were discarded as likely degraded transcripts. In addition, candidates in which potential secondary TSSs could be detected downstream of the premature terminator were discarded. Because of specific regulator degradation/processing patterns, a handful of premature terminator sites were manually corrected to a nearby, less covered term-seq site if that site presented a stem-loop and polyU signature (corrections noted in table S2). To differentiate between known and previously unidentified regulators, all candidate elements were compared against the Rfam database (33) (Rfam 11.0 2012-07-19: AL009126.3, AL591824.1, and AE016830.1 for *B. subtilis*, *L. monocytogenes*, and *E. faecalis*, respectively) and the literature. All identified candidate regulators were independently compared with the online Rfam db.

Transcriptional read-through estimation by use of RNA-seq and term-seq

Term-seq average coverage across triplicates was calculated for the premature termination site and the full-length gene termination site with a span of 10 nt surrounding each terminator, and the fraction of full-length (gene) terminations out of all termination events was used as a measure of the transcriptional read-through (Fig. 3A). In cases in which the regulator controlled the transcription of a multi-gene operon, which contained internal TSSs in addition to the primary one, RNA-seq was used to determine read-through in the first gene (Fig. 4, C and F). RNA-seq coverage was used to measure the average read coverage over either the regulatory element or the gene, and the ratio between the two (gene-coverage divided by regulator-coverage) was used as an estimate for the short/long transcript ratio generated by regulator activity (Fig. 3A).

Term-seq analysis of in vitro transcribed RNA

Term-seq libraries were prepared as above by using *L. monocytogenes* RNA spiked in with 1 µl of 1:10 diluted ERCC RNA Spike-In Mix (Ambion, 4456740). Sequencing reads were mapped to the reference ERCC sequences, and the number of term-seq reads mapped to the exact spike-in RNA 3' ends were counted and compared with the known RNA concentrations (fig. S2). Only spike-in RNAs with a known concentration of at least 1 attomoles (amol) and that were covered by at least one term-seq read ($n = 58$ RNAs) were used in the analysis.

Ribosome immunization ErmC 23S methylation

L. monocytogenes EGD-e pAT18-cGFP (53) was grown overnight in BHI media and then diluted 1:200 into fresh BHI. ErmC expression was induced by incubating the cultures with 0.5 µg/ml erythromycin for 2 hours. The induced and the control (noninduced) samples were then exposed to 0.25 µg/ml of lincomycin or water as a control and incubated for 15 min. Bacteria were harvested by means of centrifugation, flash-frozen, and the

RNA was extracted, sequenced, and analyzed as described above.

Comparative sequence analysis of *lmo0919* homologs

The *lmo0919* protein sequence was used to collect homologs in several Gram-positive bacteria (fig. S7). 5'UTRs were estimated as the 275 nt upstream top of the homologous gene and were analyzed by means of multiple sequence alignment and in silico RNA folding with Muscle (57) and with RNAfold (54), respectively. The upstream intergenic regions of *lmo0919* homologs identified in *Bacillus subtilis* subsp. *subtilis* str. 168, *Streptococcus gordonii* str. Challis subsp. CH, *Enterococcus faecalis* V583, *Staphylococcus aureus* plasmid pVGA, and *Clostridium botulinum* CDC54075 were collected by using the Integrated Microbial Genomes database (IMG) (58) using, respectively, the following IMG gene IDs: 646317030, 640912864, 637415867, 643661871, and 2569227938.

Epifluorescence analysis

A colony of wild-type EGD-e and one of EGDe-rli53-GFP mutant *Listeria* were resuspended in 20 μ L of phosphate-buffered saline and mounted on glass coverslips sealed with varnish. Samples were analyzed with an Axio Observer Z1 microscope (Zeiss) equipped with a spinning disk and an EvolveTM 512 EMCCD Camera (Photometrics). Images were acquired with a $\times$ 100 oil immersion objective and processed with MetaMorph (Universal Imaging) and Icy (Pasteur Institute).

Ribosome profiling (Ribo-seq)

L. monocytogenes EGDe and *L. innocua* Clip1262 were grown overnight in BHI media and then diluted 1:200 into fresh 200 ml BHI cultures. Cultures were grown to OD₆₀₀ = 0.4 to 5 and then treated with either 0.5 μ g/ml of lincomycin or water as a control for 15 min. The bacteria were collected via the rapid-filtration method (43) and flash-frozen in liquid nitrogen. Six hundred fifty microliters of lysis buffer containing 20 mM Tris 8.0, 10 mM MgCl₂, 100 mM NH₄Cl, 0.4% Triton X100, 0.1% NP-40, 1 mM chloramphenicol, and 100 U/ml DNase I (NEB, M0303) was dropped over liquid nitrogen and added to the pellets. Frozen cell pellets were pulverized in the MM301 Mixer mill (Retsch, Haan, Germany) by using a 10-ml stainless steel grinding jar and 12-mm grinding ball (Retsch) for five cycles, each at 30 Hz for 2 min, and chilled in liquid nitrogen between cycles. Frozen lysates were thawed on ice for 20 min and then centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatant was collected. Micrococcal nuclease (MNase) digestion was performed by mixing 1 mg RNA with CaCl₂ (5 mM final), 6 μ L Suprase-In (Ambion), and 1.7 μ L MNase (NEB, M0247). The reaction was incubated for 1 hour in 25°C with shaking and terminated by adding 5 mM EGTA (Sigma) and placing on ice. Monosomes were isolated by means of ultracentrifugation (Sw41 rotor, 35,000 rpm for 2.5 hours at 4°C) of the samples over 10 to 50% sucrose density gradient. Ribosomal footprints were collected as

described in (43). Sequencing libraries were constructed by using NEBNext Small RNA Library Prep Set for Illumina (NEB, E7330) according to the manufacturer's instructions. The sequencing reads were adapter-trimmed by using the FASTX-Toolkit (fastx_clipper -Q33 -a AGATC-GGAAGAGCACACGTCTGAACTCCAGTAC -l 25 -c -n -v -i input_fastq_file), and inserts sized 25 to 40 nt were aligned to reference genome and analyzed as above.

For each ribosome profiling sample, we also sequenced the total RNA as following: 5 μ g total RNA was fragmented as above for 5 min and then cleaned with SPRI as above and eluted in 16 μ L of RNase-free H₂O. The fragmented RNA was end-repaired by using T4-polynucleotide kinase (T4-PNK; NEB, M0201) by adding 2 μ L T4-PNK 10X buffer and 2 μ L T4-PNK and then incubating the reaction at 37°C for 2 hours. The enzyme was deactivated by incubating the sample at 75°C for 15 min, and the RNA was collected by means of isopropanol precipitation. The resulting RNA was rRNA-depleted as above, and libraries were prepared with the NEBNext Small RNA Library Prep Set for Illumina (NEB, E7330) kit as above.

Meta-term-seq

Oral plaque was collected from three healthy individuals by using a toothpick and then mixed into 1 ml of liquid BHI media, prewarmed to 37°C. The samples were vortexed for 10 s and then divided into tubes either containing BHI (control) or BHI supplemented with 1 μ g/ml lincomycin and then incubated with shaking at 37°C for 15 min. Bacteria were pelleted via centrifugation and were then flash-frozen. RNA extraction and library construction were done as described above. Sequencing reads were mapped to all contigs larger than 2.5 kb taken from the 462 annotated genomes available at the Human Oral Microbiome Database (HOMD) (46). In cases in which multiple closely related strains of a single species were present in the database, only one representative genome was selected. Only bacterial genomes in which at least 10% of all protein-coding genes were covered by 10 or more uniquely mapped reads were selected for further analysis. Sequencing reads were remapped to the final database of 167 bacterial genomes. Term-seq and 5' end sequencing reads were mapped by using the novoalign-r Random option. To identify lincomycin-responsive regulators, genes with a mapped TSS and premature term-seq site and that were covered by $\geq$ 50 RNA-seq reads and showed a $>$ 2.5-fold increase in expression in the lincomycin condition were selected for read-through calculation. Candidate regulators were classified as lincomycin-responsive if they increased their read-through by at least 2.5-fold after exposure to lincomycin and that had a read-through $>$ 10% in the lincomycin condition.

Construction of the phylogenetic tree in Fig. 6B was performed by using 16S rRNA sequences, taken from the Human Oral Microbiome Database (HOMD) or National Center for Biotechnology Information (NCBI), and by using the *phylogenyfr* Web server with default parameters (59). The

phylogenetic tree was deposited in the treebase online database (treebase.org) under accession no. 18921.

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S11

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RESEARCH ARTICLES

CANCER GENOMICS

Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq

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To explore the distinct genotypic and phenotypic states of melanoma tumors, we applied single-cell RNA sequencing (RNA-seq) to 4645 single cells isolated from 19 patients, profiling malignant, immune, stromal, and endothelial cells. Malignant cells within the same tumor displayed transcriptional heterogeneity associated with the cell cycle, spatial context, and a drug-resistance program. In particular, all tumors harbored malignant cells from two distinct transcriptional cell states, such that tumors characterized by high levels of the MITF transcription factor also contained cells with low MITF and elevated levels of the AXL kinase. Single-cell analyses suggested distinct tumor microenvironmental patterns, including cell-to-cell interactions. Analysis of tumor-infiltrating T cells revealed exhaustion programs, their connection to T cell activation and clonal expansion, and their variability across patients. Overall, we begin to unravel the cellular ecosystem of tumors and how single-cell genomics offers insights with implications for both targeted and immune therapies.

Tumors are complex ecosystems defined by spatiotemporal interactions between heterogeneous cell types, including malignant, immune, and stromal cells (1). Each tumor's cellular composition, as well as the interplay between these components, may exert critical roles in cancer development (2). However, the specific components, their salient biological functions, and the means by which they collectively define tumor behavior remain incompletely characterized.

Tumor cellular diversity poses both challenges and opportunities for cancer therapy. This is exemplified by the varied clinical efficacy achieved in malignant melanoma with targeted therapies and immunotherapies. Immune checkpoint inhibitors can produce clinical responses in many patients with metastatic melanomas (3–7); how-

ever, the genomic and molecular determinants of response to these agents remain incompletely understood. Although tumor neoantigens and PD-L1 expression clearly correlate with this response (8–10), it is likely that other factors from subsets of malignant cells, the microenvironment, and tumor-infiltrating lymphocytes (TILs) also play essential roles (11).

Melanomas that harbor the *BRAF*^{V600E} (V600E: Val⁶⁰⁰→Glu⁶⁰⁰) mutation are commonly treated with inhibitors of rapidly accelerated fibrosarcoma kinase (RAF) and mitogen-activated protein kinase (MEK), before or after immune checkpoint inhibition. Although this regimen improves survival, virtually all tumors eventually develop resistance to these drugs (12, 13). Unfortunately, no targeted therapy currently exists for patients

whose tumors lack *BRAF* mutations—including *NRAS* mutant tumors, those with inactivating *NF1* mutations, or rarer events (such as *RAF* fusions). Collectively, these factors highlight the need for a deeper understanding of melanoma composition and its effect on the clinical course.

The next wave of therapeutic advances in cancer will probably be accelerated by technologies that assess the malignant, microenvironmental, and immunologic states most likely to inform treatment response and resistance. Ideally, we would be able to assess salient cellular heterogeneity by quantifying variation in oncogenic signaling pathways; drug-resistant tumor cell subsets; and the spectrum of immune, stromal, and other cell states that may inform immunotherapy response. Toward this end, single-cell genomic approaches enable detailed evaluation of genetic and transcriptional features present in hundreds to thousands of individual cells per tumor (14–16). In principle, this approach may allow us to identify all major cellular components simultaneously, determine their individual genomic and molecular states (15), and ascertain which of these features may predict or explain clinical responses to anticancer agents. To explore this question, we used single-cell RNA sequencing (RNA-seq) to examine heterogeneities in malignant and nonmalignant cell types and states and to infer their possible drivers and interrelationships in the complex tumor cellular ecosystem.

Profiles of individual cells from patient-derived melanoma tumors

We measured single-cell RNA-seq profiles from 4645 malignant, immune, and stromal cells isolated from 19 freshly procured human melanoma tumors that span a range of clinical and therapeutic backgrounds (table S1). These included 10 metastases to lymphoid tissues (9 to lymph nodes and 1 to the spleen), 8 to distant sites (5 to subcutaneous or intramuscular tissue and 3 to the gastrointestinal tract), and one primary acral melanoma. Genotypic information was available for 17 of the 19 tumors, of which 4 had activating mutations in *BRAF* and 5 in *NRAS* oncogenes; eight patients had *BRAF*/*NRAS* wild-type melanomas (table S1).

To isolate viable single cells that are suitable for high-quality single-cell RNA-seq, we developed and implemented a rapid translational workflow (Fig. 1A) (15). We processed tumor tissues immediately after surgical procurement and generated single-cell suspensions within ~45 min, using an experimental protocol optimized to reduce artifactual transcriptional changes introduced by disaggregation, temperature, or time (17). Once in

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suspension, we recovered individual viable immune (CD45⁺) and nonimmune (CD45⁻) cells (including malignant and stromal cells) by flow cytometry (fluorescence-activated cell sorting). Next, we prepared cDNA from the individual cells, followed by library construction and massively parallel sequencing. The average number of mapped reads per cell was ~150,000 (17), with a median library complexity of 4659 genes for malignant cells and 3438 genes for immune cells, comparable to previous studies of only malignant cells from fresh glioblastoma tumors (15).

Single-cell transcriptome profiles distinguish cell states in malignant and nonmalignant cells

We used a multistep approach to distinguish the different cell types within melanoma tumors

on the basis of both genetic and transcriptional states (Fig. 1, B to D). First, we inferred large-scale copy number variations (CNVs) from expression profiles by averaging expression over stretches of 100 genes on their respective chromosomes (15) (Fig. 1B). For each tumor, this approach revealed a common pattern of aneuploidy, which we validated in two tumors by bulk whole-exome sequencing (WES) (Fig. 1B and fig. S1A). Cells in which aneuploidy was inferred were classified as malignant cells (Fig. 1B and fig. S1).

Second, we grouped the cells according to their expression profiles (Fig. 1, C and D, and fig. S2). To do this, we used nonlinear dimensionality reduction [t-distributed stochastic neighbor embedding (t-SNE)] (18), followed by density clustering (19). Generally, cells designated as

malignant by CNV analysis formed a separate cluster for each tumor (Fig. 1C), suggesting a high degree of intertumor heterogeneity. In contrast, the nonmalignant cells clustered by cell type (Fig. 1D and fig. S2), independent of their tumor of origin and metastatic site (fig. S3). Clusters of nonmalignant cells were annotated as T cells, B cells, macrophages, endothelial cells, cancer-associated fibroblasts (CAFs), and natural killer cells on the basis of their preferentially or distinctively expressed marker genes (Fig. 1D, fig. S2, and tables S2 and S3).

Analysis of malignant cells reveals heterogeneity in cell cycle and spatial organization

We next used unbiased analyses of the individual malignant cells to identify biologically relevant

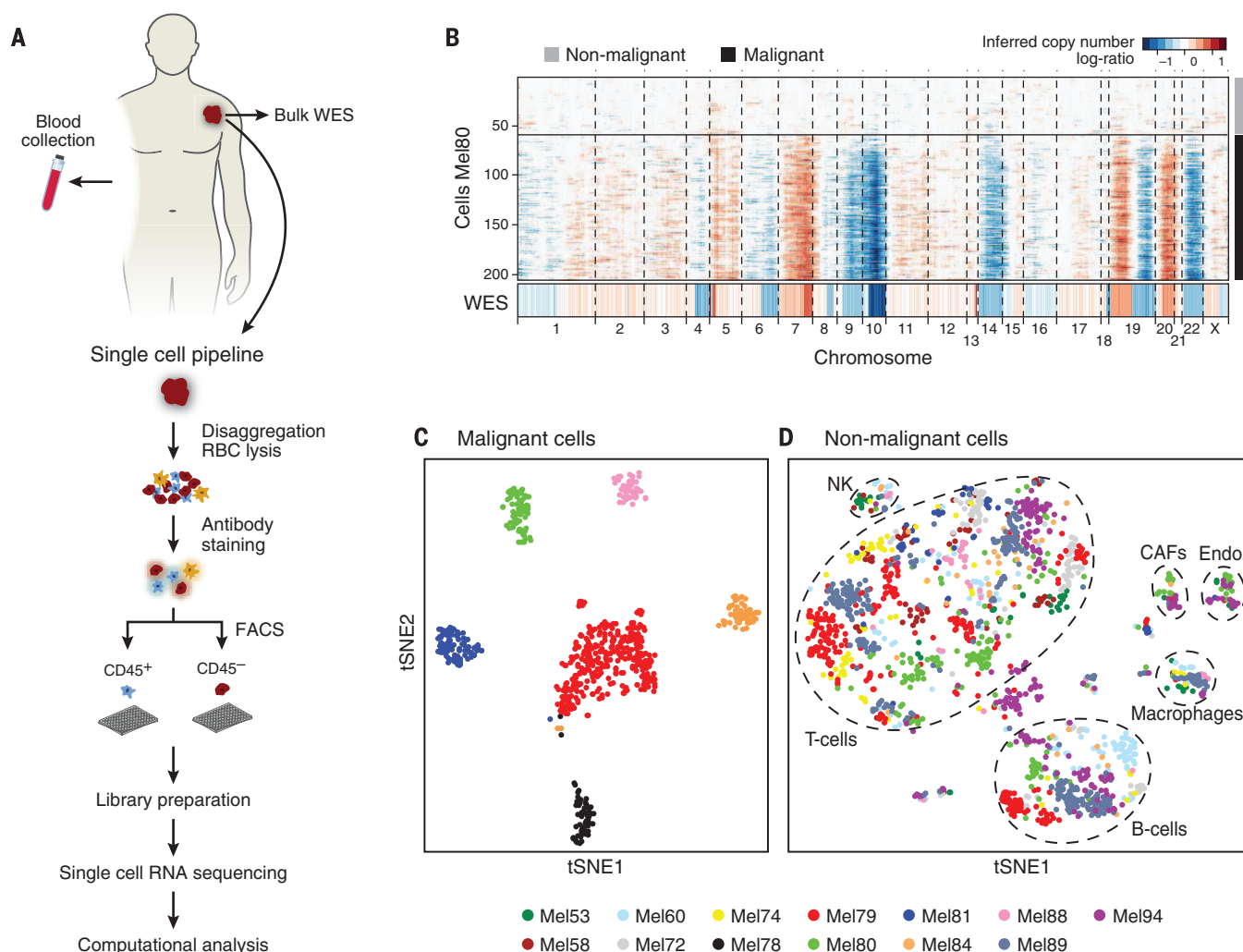


Fig. 1. Dissection of melanoma with single-cell RNA-seq. (A) Overview of workflow. WES, whole-exome sequencing; RBC, red blood cell; FACS, fluorescence-activated cell sorting. (B) Chromosomal landscape of inferred large-scale CNVs allows us to distinguish malignant from nonmalignant cells. The Mel80 tumor is shown with individual cells (y axis) and chromosomal regions (x axis). Amplifications (red) or deletions (blue) were inferred by averaging expression over 100-gene stretches on the respective chromosomes. Inferred CNVs are concordant with calls from WES (bottom). (C and D) Single-cell expression profiles allow

us to distinguish malignant and nonmalignant cell types. Shown are t-SNE plots of malignant [(C), shown are the six tumors, each with >50 malignant cells] and nonmalignant (D) cells [as called from inferred CNVs as in (B)] from 11 tumors with >100 cells per tumor (see color code below the panels). Clusters of non-malignant cells [called by DBScan (17, 19)] are marked by dashed ellipses and were annotated as T cells, B cells, macrophages, CAFs, and endothelial (Endo.) cells from preferentially expressed genes (fig. S2 and tables S2 and S3). NK, natural killer cells.

melanoma cell states. After controlling for inter-tumor differences (17), we examined the six top components from a principal component analysis (PCA) (table S4). The first component correlated highly with the number of genes detected per cell and probably reflects technical aspects, whereas the other five significant principal components highlighted biological variability.

The second component (PC2) was associated with the expression of cell cycle genes (Gene On-

tology project: “cell cycle” $P < 10^{-16}$; hypergeometric test). To characterize cycling cells more precisely, we used gene signatures that have previously been shown to denote G₁/S or G₂/M phases in both synchronization (20) and single-cell (16) experiments in cell lines. Cell cycle phase-specific signatures were highly expressed in a subset of malignant cells, distinguishing cycling cells from noncycling cells (Fig. 2A and fig. S4A). These signatures revealed

variability in the fraction of cycling cells across tumors (13.5% on average, ± 13 SD) (fig. S4B), allowing us to designate both low-cycling (1 to 3%, e.g., Mel79) and high-cycling tumors (20 to 30%, e.g., Mel78), consistent with *Ki67*⁺ staining results (Fig. 2B and fig. S4C).

A core set of cell cycle genes was induced (fig. S4D, red dots; and table S5) in both low-cycling and high-cycling tumors, with one notable exception: *cyclin D3* (*CCND3*), which was induced in cycling cells only in high-cycling tumors (fig. S4D). In contrast, *KDM5B* (*JARID1B*) showed the strongest association with noncycling cells (Fig. 2A, green dots), mirroring Patel *et al.*'s findings in glioblastoma (15). *KDM5B* encodes a H3K4 histone demethylase associated with a subpopulation of slow-cycling and drug-resistant melanoma stemlike cells (21, 22) in mouse models. Immunofluorescence (IF) staining validated the presence and mutually exclusive expression of KDM5B and Ki67. KDM5B-expressing cells were grouped in small clusters, consistent with observations in mouse and in vitro models (21) (Fig. 2C and fig. S4E).

Two principal components (PC3 and PC6) primarily segregated different malignant cells from one treatment-naïve tumor (Mel79). In this tumor, we analyzed 468 malignant cells from four distinct regions after surgical resection (fig. S5A). We identified 229 genes with higher expression in the malignant cells of region one compared with those of other tumor regions [Fig. 2D, false discovery rate (FDR) < 0.05; and table S6]. A similar expression program was found in T cells from region one (fig. S6 and table S6), suggesting a spatial effect that influences multiple cell types. The genes with the highest preferential expression in region one are also generally coexpressed across melanoma tumors profiled in bulk in The Cancer Genome Atlas (TCGA) (23) (fig. S6). Many of these genes encode immediate early-activation transcription factors linked to inflammation, stress responses, and a melanoma oncogenic program (e.g., *ATF3*, *FOS*, *FOSB*, *JUN*, *JUNB*). Several of these transcription factors (e.g., *FOS*, *JUN*, *NR4A1/2*) are regulated by cyclic adenosine monophosphate (cAMP) and cAMP response element-binding protein signaling, which has been implicated as a mitogen-activated protein kinase (MAPK)-independent resistance module in *BRAF*-mutant melanomas treated with RAF and MEK inhibition (24). Other top genes differentially up-regulated in region one included those involved in survival (*MCL1*), stress responses (*EGR1/2/3*, *NDRG*, *HSPA1B*), and NF- κ B signaling (*NFKB1Z*), which has also been associated with resistance to RAF and MEK inhibition (25). Immunohistochemistry analysis confirmed the elevated NF- κ B and JunB levels in cells of region one compared with cells in the other regions of this tumor (fig. S5B).

Heterogeneity in the abundance of a dormant, drug-resistant melanoma subpopulation

Collectively, the above observations imply that pretreatment melanoma tumors may harbor

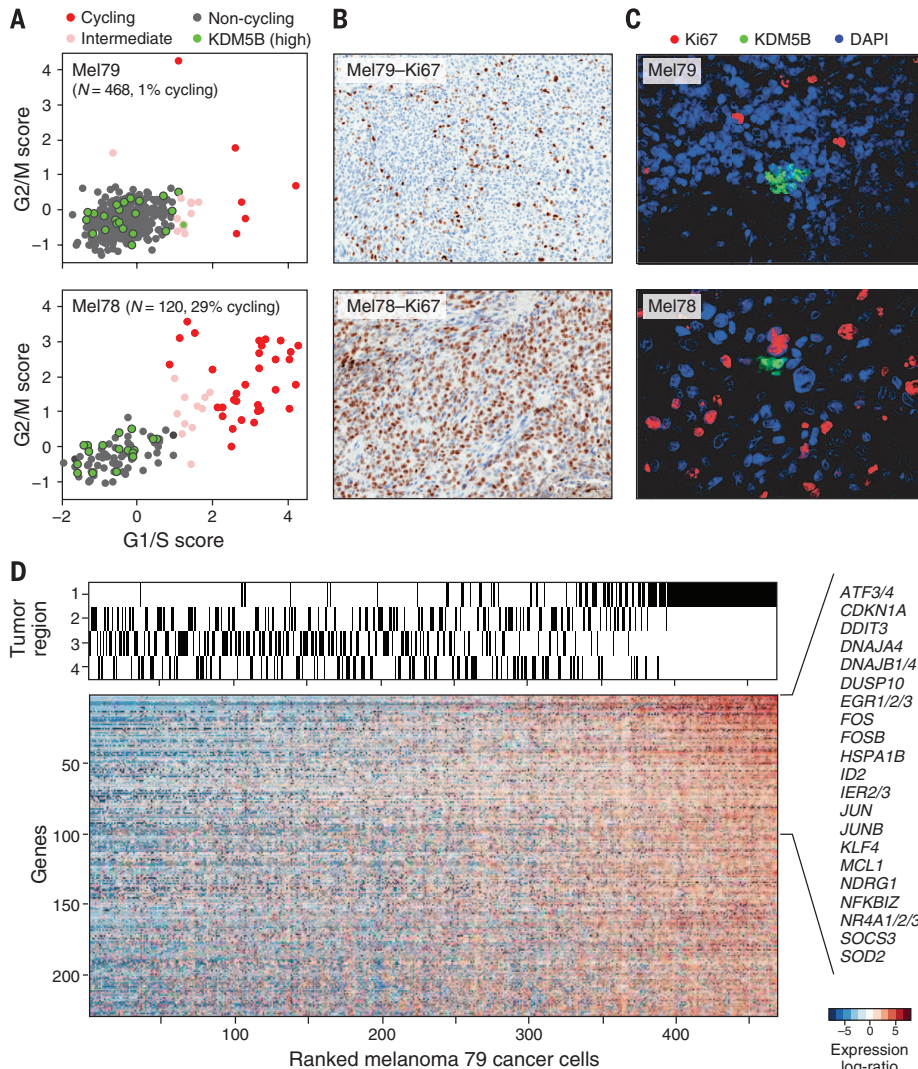


Fig. 2. Single-cell RNA-seq distinguishes cell cycle and other states among malignant cells. (A) Estimation of the cell cycle state of individual malignant cells (circles) on the basis of relative expression of G₁/S (x axis) and G₂/M (y axis) gene sets in a low-cycling tumor (Mel79, top) and a high-cycling tumor (Mel78, bottom). Cells are colored by their inferred cell cycle states: cycling cells, red; intermediate, pink; and noncycling cells, gray. Cells with high expression of *KDM5B* (z score > 2) are shown in green. *N* denotes number of cells. (B) Immunohistochemistry staining (40× magnification) for *Ki67*⁺ cells shows concordance with the signature-based frequency of cycling cells for Mel79 and Mel78 (as for other tumors; fig. S4C). (C) KDM5B and Ki67 staining (40× magnification) in corresponding tissue showing small clusters of KDM5B-high expressing cells negative for Ki67 (fig. S4). DAPI, 4',6-diamidino-2-phenylindole. (D) An expression program specific to region one of Mel79, identified on the basis of multifocal sampling. The relative expression of genes (rows) is shown for cells (columns) ordered by the average expression of the entire gene set. The region of origin of each cell is indicated in the top panel (fig. S5).

subsets of malignant cells that are less likely to respond to targeted therapy. The transcriptional programs associated with principal components PC4 and PC5 were highly correlated with expression of the *MITF* gene (*microphthalmia-associated transcription factor*), which encodes the master melanocyte transcriptional regulator and a melanoma lineage-survival oncogene (26). Scoring genes by their correlation to *MITF* across single cells, we identified a “MITF-high” program consisting of *MITF* itself and several MITF target genes, including *TYR*, *PMEL*, and *MLANA* (table S7). A second transcriptional program, negatively correlated with the MITF program and with PC4 and PC5 (Pearson correlation $P < 10^{-24}$), included *AXL* and *NGFR* (p75NTR), a marker of resistance to various targeted therapies (27, 28) and a putative melanoma cancer stem cell marker (29), respectively (table S8). Thus, these transcriptional programs re-

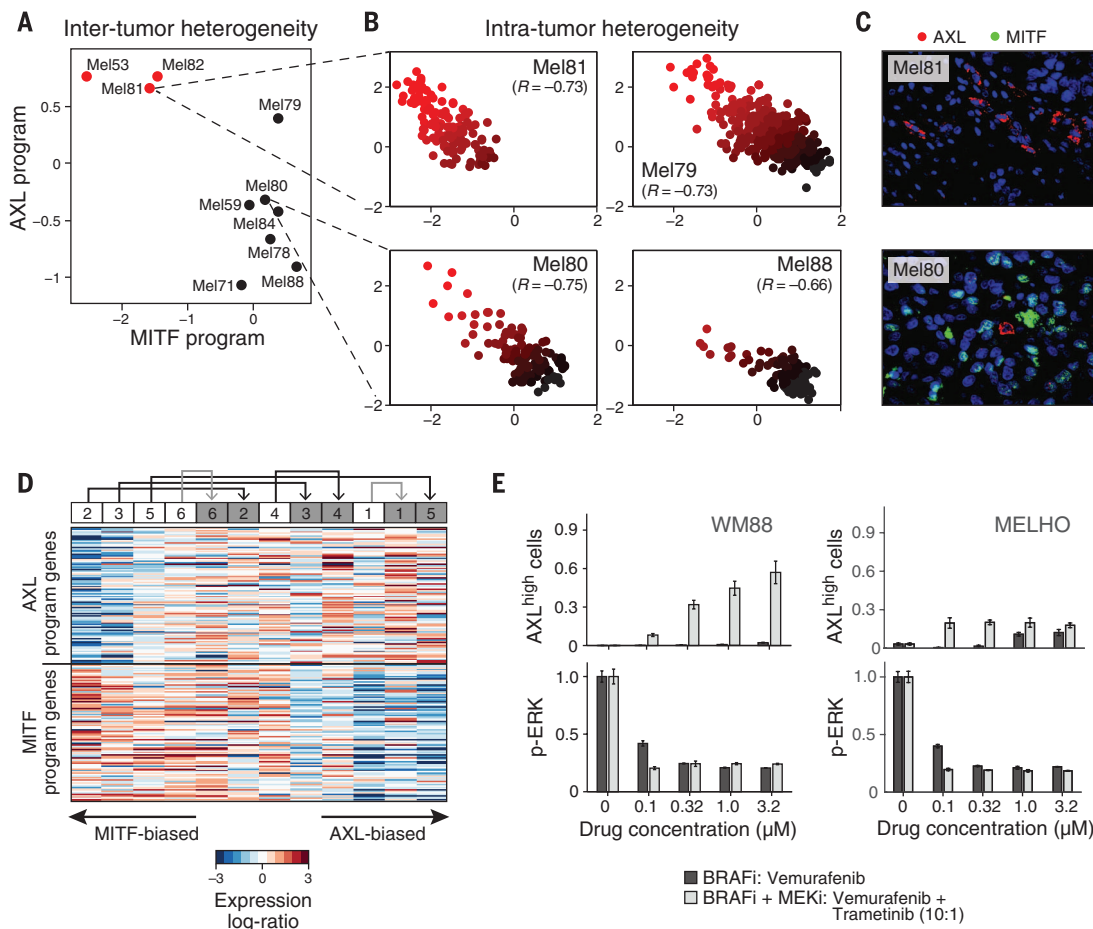
semble reported (25, 30–32) MITF-high, as well as MITF-low and AXL-high (“AXL-high”), transcriptional profiles that can distinguish melanoma tumors, cell lines, and mouse models. Notably, the AXL-high program has been linked to intrinsic resistance to RAF and MEK inhibition (25, 30, 31).

Although at the bulk tumor level each melanoma could be classified as MITF-high or AXL-high (Fig. 3A), at the single-cell level every tumor contained malignant cells corresponding to both transcriptional states. Using single-cell RNA-seq to examine each cell's expression of the MITF and AXL gene sets, we observed that MITF-high tumors, including treatment-naïve melanomas, harbored a subpopulation of AXL-high melanoma cells that was undetectable through bulk analysis, and vice versa (Fig. 3B). The malignant cells thus spanned the continuum between AXL-high and MITF-high states in every investigated

tumor (Fig. 3B and fig. S7). We performed IF staining to further validate the mutually exclusive expression of the MITF-high and AXL-high programs in cells from the same bulk tumors (Fig. 3C and fig. S8).

Because malignant cells with AXL-high and MITF-high transcriptional states coexist in melanoma, we hypothesized that treatment with RAF and MEK inhibitors would increase the prevalence of AXL-high cells after the development of drug resistance. To test this, we analyzed RNA-seq data from a cohort (13) of six paired *BRAF*^{V600E} melanoma biopsies taken before treatment and after resistance to single-agent RAF inhibition (vemurafenib; 1 patient) or combined RAF and MEK inhibition (dabrafenib and trametinib; 5 patients), respectively (tables S9 and S10). We ranked the 12 transcriptomes on the basis of the relative expression of all genes in the AXL-high program compared with those in the

Fig. 3. MITF- and AXL-associated expression programs vary between and within tumors, as well as after treatment. (A) Average expression signatures for the AXL program (y axis) or the MITF program (x axis) stratify tumors into MITF-high (black) or AXL-high (red) categories. (B) Single-cell profiles show a negative correlation between the AXL program (y axis) and the MITF program (x axis) across individual malignant cells within the same tumor. Cells are colored by the relative expression of the MITF (black) and AXL (red) programs. Cells in both states are found in all examined tumors, including three tumors (Mel79, Mel80, and Mel81) without prior systemic treatment, indicating that dormant resistant (AXL-high) cells may be present in treatment-naïve patients. (C) Mel81 and Mel80 IF staining of MITF (green nuclei) and AXL (red), validating the mutual exclusivity among individual cells within the same tumor (fig. S8). (D) Relative expression



(centered) of the AXL program genes (top) and MITF program genes (bottom) in six matched pretreatment (white boxes) and postrelapse (gray boxes) samples from patients who progressed through therapeutic RAF and MEK inhibition. Numbers at the top indicate patient index. Samples are sorted by the average relative expression of the AXL versus MITF gene sets. In all cases, the relapsed samples had an increased ratio of AXL-to-MITF expression compared with their pretreatment counterparts. This consistent shift of all six patients is statistically significant ($P < 0.05$, binomial test), as are the individual increases in AXL and MITF for four of the six sample pairs ($P < 0.05$, t test;

black and gray arrows denote increases that are individually significant or nonsignificant, respectively). (E) Quantitative, multiplexed single-cell IF for AXL expression (top y axes) and MAP kinase pathway inhibition (p-ERK levels, bottom y axes) in the example cell lines WM88 and MELHO treated with increasing concentrations (x axis) of either a RAF inhibitor alone (dark gray bars) or a combination of RAF and MEK inhibitors (light gray bars). We observed an increasing fraction of AXL-high cells (top panels) as well as a dose-dependent decrease of p-ERK (bottom panels) (figs. S11 and S12 show results for additional cell lines).

MITF-high program. In each pair, we observed a shift toward the AXL-high program in the drug-resistant sample [Fig. 3D; $P < 0.05$ for same effect in six of six paired samples, binomial test; $P < 0.05$ for four of six individual paired-sample comparisons shown by black arrows (17)]. RNA-seq data from an independent cohort (33) also showed that a subset of drug-resistant samples exhibited increased expression of the AXL program (fig. S9). Other genes previously

implicated in resistance to RAF and MEK inhibition were also increased in a subset of the drug-resistant samples. PDGFRB (platelet-derived growth factor receptor β) (34) was up-regulated in a similar subset as the AXL program, whereas MET (33) was up-regulated in a mutually exclusive subset (fig. S9), suggesting that AXL and MET may reflect distinct drug-resistant states.

To further assess the connection between the AXL program and resistance to RAF and MEK

inhibition, we studied single-cell AXL expression in 18 melanoma cell lines from the Cancer Cell Line Encyclopedia (35) (table S11). Flow cytometry analysis revealed a wide distribution of the proportion of AXL-positive cells, from <1 to 99% per cell line, which correlated with bulk mRNA levels and was inversely associated with sensitivity to small-molecule RAF inhibition (table S11).

We treated 10 cell lines (17) with increasing doses of a combination of RAF and MEK inhibitors

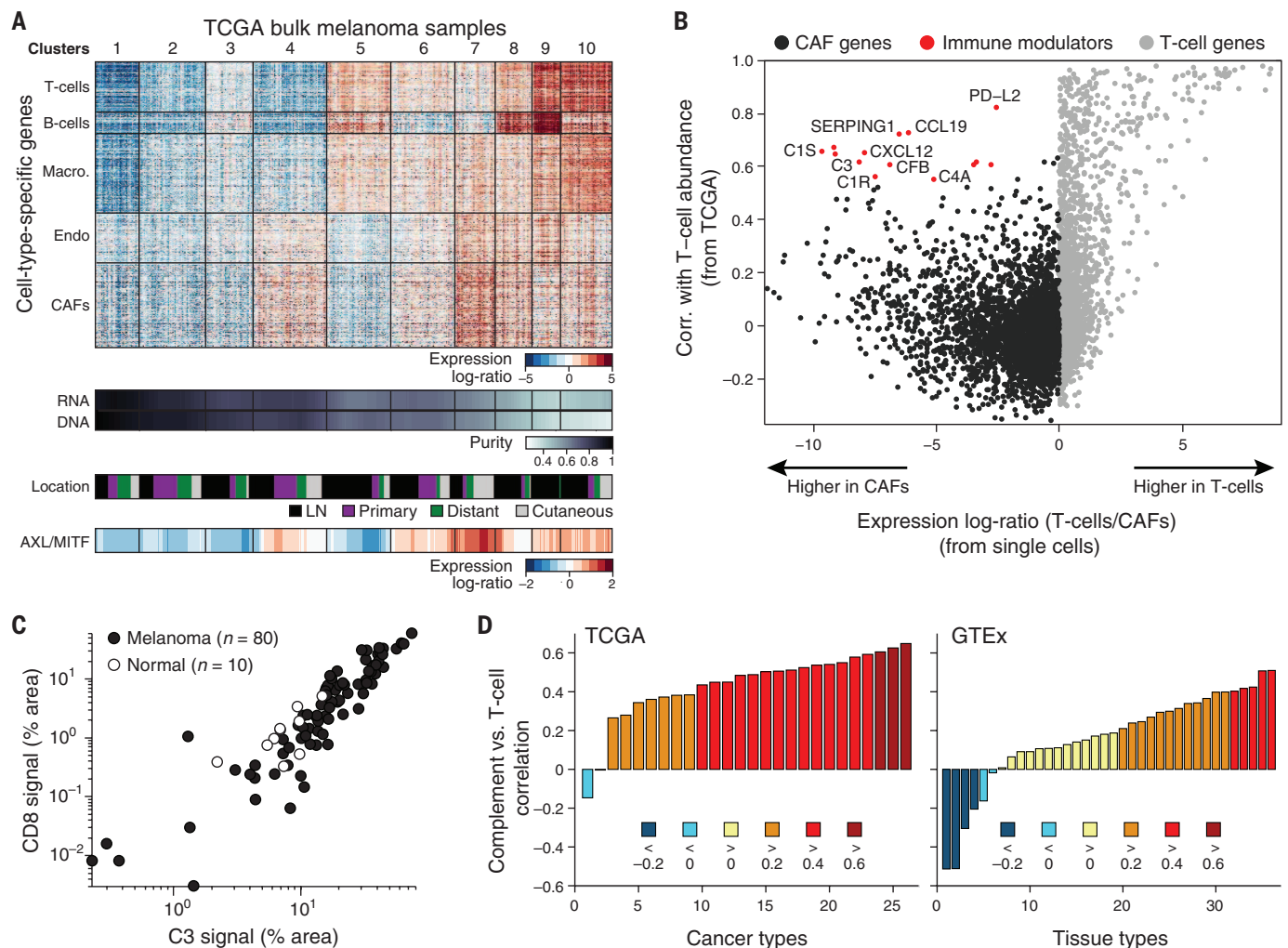


Fig. 4. Deconvolution of bulk melanoma profiles reveals cell-to-cell interactions. (A) Bulk tumors segregate to distinct clusters on the basis of their inferred cell type composition. (Top panel) Heat map showing the relative expression of gene sets defined from single-cell RNA-seq, as specific to each of five cell types from the tumor microenvironment (y axis) across 471 melanoma TCGA bulk-RNA signatures (x axis). Each column represents one tumor, and tumors are partitioned into 10 distinct patterns identified by *k*-means clustering (vertical lines and cluster numbers at the top). Endo, endothelial cells; Macro., macrophages. (Lower panels, from top to bottom) Tumor purity estimated by ABSOLUTE (DNA) and RNA-seq analysis (RNA), specimen location (from TCGA), and AXL/MITF scores. Tumors with a high abundance of CAFs are correlated with an increased ratio of AXL-to-MITF expression (bottom). LN, lymph node. (B) Inferred cell-to-cell interactions between CAFs and T cells. The scatter plot compares, for each gene (circle), the correlation of its expression with inferred T cell abundance across bulk

tumors (y axis, from TCGA transcriptomes) to the specificity of its expression in CAFs (black) versus T cells (gray) (x axis, based on single-cell transcriptomes). Genes that are highly specific to CAFs in a single-cell analysis of tumors but are also associated with high T cell abundance in bulk tumors (red) are candidates for interaction between CAF cells and T cells. (C) Of the 90 samples, 80 tumor specimens (black dots) show a correlation ($R = 0.86$) between C3 and CD8 signals, as analyzed by quantitative IF. Ten normal control specimens (gray dots) are also shown (fig. S18, A to F, shows normalization and additional specimens). (D) Correlation coefficient (y axis) between the average expression of CAF-derived complement factors shown in (B) and that of T cell markers (CD3/D/E/G, CD8A/B) across 26 TCGA cancer types with >100 samples (x axis, left panel) and across 36 GTEx (Genotype-Tissue Expression Project) tissue types with >100 samples (x axis, right panel). Bars are colored on the basis of correlation ranges, as indicated at the bottom.

(dabrafenib and trametinib) and found an increase in the proportion of AXL-positive cells in 6 cell lines initially composed of a small (<3%) pretreatment AXL-positive population (fig. S10A). In contrast, cell lines with an intrinsically high

proportion of AXL expression showed modest or no changes (fig. S10B). We obtained similar results by multiplexed quantitative single-cell IF, which also demonstrated that the increased fraction of AXL-positive cells after inhibition of

RAF and MEK is associated with rapid decreases in extracellular signal-regulated kinase (ERK) phosphorylation (reflecting MAP kinase signaling inhibition) (Fig. 3E and figs. S11 and S12). In summary, both melanoma tumors and cell

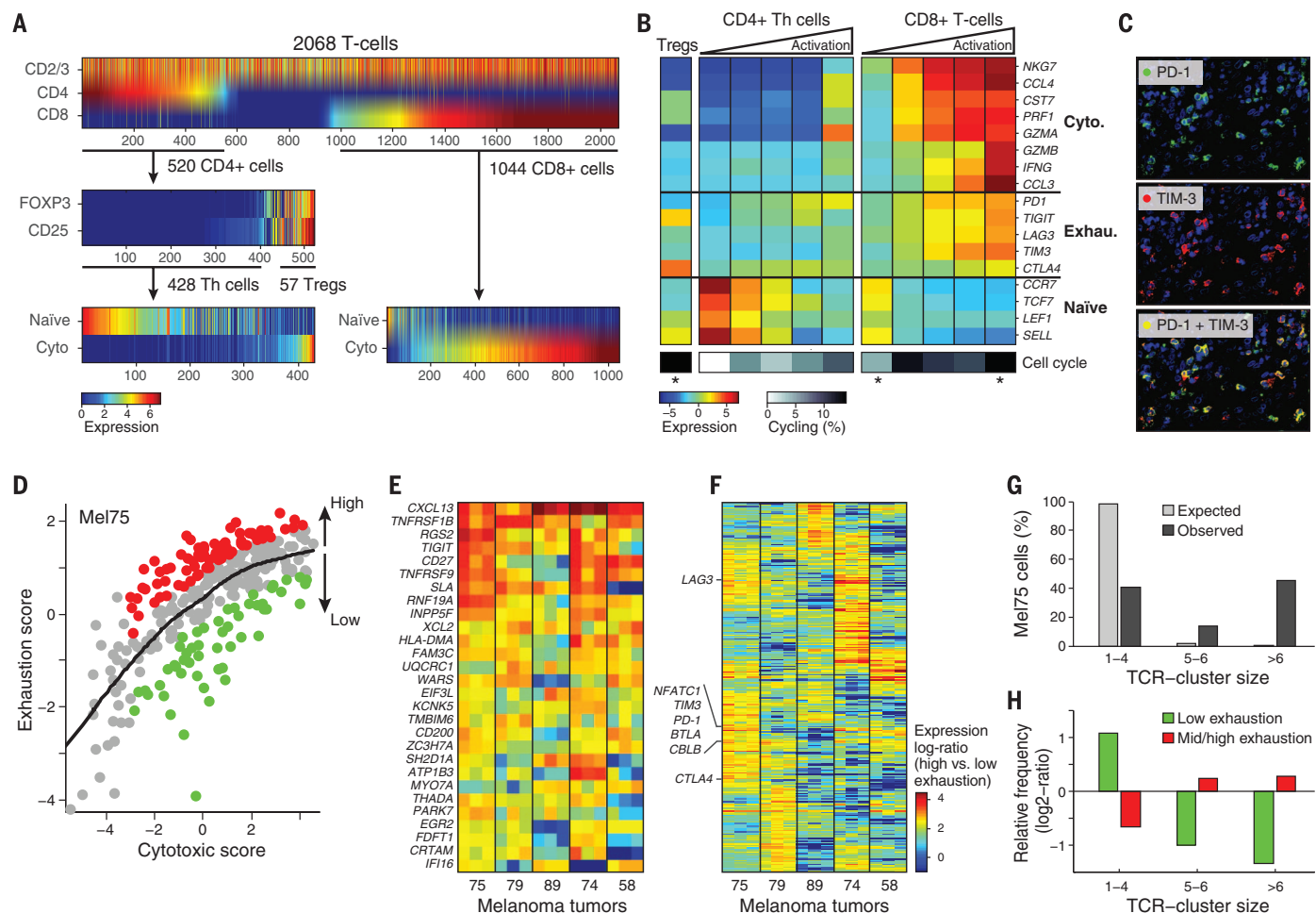


Fig. 5. Activation-dependent and -independent variation in T cell exhaustion markers. (A) Single T cell stratification into CD4⁺ and CD8⁺ cells (top panel), CD25⁺FOXP3⁺ and other CD4 cells (middle panel), and their inferred activation state [lower panels, from average expression of the cytotoxic and naïve gene sets in (B)]. Th, T helper cells; Tregs, regulatory T cells. (B) (Top) Average expression of markers of cytotoxicity (Cyto.), exhaustion (Exhau.), and naïve cell states (rows) in (from left to right) T_{regs}, CD4⁺ T helper cells, and CD8⁺ T cells. CD4⁺ and CD8⁺ T cells are each further divided into five bins by their cytotoxic score (ratio of cytotoxic to naïve marker expression levels), showing activation-dependent coexpression of exhaustion markers. (Bottom) Proportion of cycling cells (calculated as in Fig. 2B). Asterisks denote significant enrichment or depletion of cycling cells in a specific subset, as compared with the corresponding set of CD4⁺ or CD8⁺ T cells ($P < 0.05$, hypergeometric test). (C) IF analysis of PD-1 (top, green), TIM-3 (middle, red), and their overlay (bottom) validates their coexpression. (D) Activation-independent variation in exhaustion states within highly cytotoxic T cells. The scatter plot shows the cytotoxic score (x axis) and exhaustion score (y axis), average expression of the Mel75 exhaustion program as in fig. S21) of each CD8⁺ T cell from Mel75. In addition to the overall correlation between cytotoxicity and exhaustion, the cytotoxic cells can be subdivided into cells with high (red) and low exhaustion (green), based on comparison to a LOWESS (locally weighted scatter plot smoothing) regression (black line).

(E and F) Relative expression (log₂ fold-change) in high- versus low-exhaustion cytotoxic CD8⁺ T cells from five tumors (x axis), including 28 genes that were significantly up-regulated ($P < 0.05$, permutation test) in high-exhaustion cells across most tumors (E) and 272 genes that were variably associated with high-exhaustion cells across tumors (F). Three independently derived exhaustion gene sets were used to define high- and low-exhaustion cells (Mel75) (17, 46, 48), and the corresponding results are represented as distinct columns for each tumor. (G) Expanded TCR clones. Cells were assigned to clusters of TCR segment usage (dark gray bars) (fig. S23), and cluster size (x axis) was evaluated for significance by control analysis in which TCR segments were shuffled across cells (light gray bars). The percentage of Mel75 cells (y axis) is shown for clusters of small size (one to four cells) that probably represent nonexpanded cells, medium size (five or six cells) that may reflect expanded clones (FDR = 0.12), and large size (more than six cells) that most likely reflect expanded clones (FDR = 0.005). (H) Expanded clones are depleted of nonexhausted cells and enriched for exhausted cells. Mel75 cells were divided according to exhaustion score into categories of low exhaustion (green, bottom 25% of cells) and medium-to-high exhaustion (red, top 75%). Shown is the relative frequency of these exhaustion subsets (y axis) in each TCR-cluster group [x axis, as defined in (G)], defined as the log₂ ratio of the frequency in that group compared with the frequency across all Mel75 cells. All values were significant ($P < 10^{-5}$, binomial test).

lines demonstrate drug-resistant tumor cell subpopulations that precede treatment and become enriched after MAP kinase-targeted treatment.

Nonmalignant cells and their interactions within the melanoma microenvironment

Various nonmalignant cells make up the tumor microenvironment. The composition of the microenvironment has an important effect on tumorigenesis and also in the modulation of treatment responses (1). Tumor infiltration with T cells, for example, is predictive for the response to immune checkpoint inhibitors in various cancer types (36).

To resolve the composition of the melanoma microenvironment, we used our single-cell RNA-seq profiles to define distinct expression signatures of each of five distinct nonmalignant cell types: T cells, B cells, macrophages, endothelial cells, and CAFs. Because our signatures were derived from single-cell profiles, we could avoid confounders and ensure that each signature is determined by cell type-specific profiles (17). Next, we used these signatures to infer the relative abundance of those cell types in a larger compendium of tumors (17) (Fig. 4A and fig. S13). We found a strong correlation (correlation coefficient $R > 0.8$) between our estimated tumor purity and that predicted from DNA analysis (37) (Fig. 4A, first lane below the heat map).

We partitioned 471 tumors from TCGA into 10 distinct microenvironment clusters on the basis of their inferred cell type composition (Fig. 4A). Clusters were mostly independent of the site of metastasis (Fig. 4A, second lane), with some exceptions (e.g., clusters 8 and 9). Next we examined how these different microenvironments may relate to the phenotype of the malignant cells. In particular, CAF abundance is predictive of the AXL-MITF distinction, with CAF-rich tumors expressing the AXL-high signature (Fig. 4A, bottom lane). Interestingly, an AXL-high program was expressed by both melanoma cells and CAFs. However, we distinguished AXL-high genes that are preferentially expressed by melanoma cells ("melanoma-derived AXL program") from those that are preferentially expressed by CAFs ("CAF-derived AXL program"). Both sets of genes were correlated with the inferred CAF abundance in tumors from TCGA (fig. S14) (38). Furthermore, the MITF-high program, which is specific to melanoma cells, was negatively correlated with inferred CAF abundance. Taken together, these results suggest that CAF abundance may be linked to preferential expression of the AXL-high over the MITF-high program by melanoma cells. Thus it is possible that specific tumor-CAF interactions may shape the melanoma cell transcriptome.

Interactions between cells play crucial roles in the tumor microenvironment (1). To assess how cell-to-cell interactions may influence tumor composition, we searched for genes expressed by cells of one type that may influence or reflect the proportion of cells of a different type in the tumor (fig. S15). For example, we searched for genes expressed primarily by CAFs (but not T cells) in single-cell data that correlated with T cell abun-

dance (as inferred by T cell-specific genes) in bulk tumor tissue from the TCGA data set (23). We identified a set of CAF-expressed genes that correlated strongly with T cell infiltration (Fig. 4B, red circles). These included known chemotactic (*CXCL12* and *CCL19*) and immune-modulating (*PD-L2*) genes, which are expressed by both CAFs and macrophages (fig. S16). A separate set of genes, exclusively expressed by CAFs, that correlated with T cell infiltration (fig. S16) included multiple complement factors [*C1S*, *C1R*, *C3*, *C4A*, *CFB*, and *CINH* (*SERPIN1*)]. Notably, these complement genes were specifically expressed by freshly isolated CAFs but not by cultured CAFs (fig. S17) or macrophages (fig. S16). These findings are intriguing, as studies have implicated complement activity in the recruitment and modulation of T cell-mediated antitumor immune responses [in addition to their role in augmenting innate immunity (39)].

We validated a high correlation ($R > 0.8$) between complement factor 3 (C3) levels (one of the CAF-expressed complement genes) and infiltration of CD8⁺ T cells. We performed dual IF staining and quantitative slide analysis of two tissue microarrays with a total of 308 core biopsies, including primary tumors, metastatic lesions, normal skin with adjacent tumor, and healthy skin controls (Fig. 4C and fig. S18) (17). To test the generalizability of the association between CAF-derived complement factors with T cell infiltration, we expanded our analysis to bulk RNA-seq data sets across all TCGA cancer types (Fig. 4D). Consistent with the results in melanoma, complement factors correlated with inferred T cell abundance in many cancer types and more highly than in normal tissues (e.g., $R > 0.4$ for 65% of cancer types but only for 14% of normal tissue types). Although correlation analysis cannot determine causality, this indicates a potential *in vivo* role for cell-to-cell interactions.

Diversity of tumor-infiltrating T lymphocytes and their functional states

The activity of TILs, particularly CD8⁺ T cells, is a major determinant of successful immune surveillance. Under normal circumstances, effector CD8⁺ T cells exposed to antigens and costimulatory factors may mediate lysis of malignant cells and control tumor growth. However, this function can be hampered by tumor-mediated T cell exhaustion, such that T cells fail to activate cytotoxic effector functions (40). Exhaustion is promoted through the stimulation of coinhibitory checkpoint molecules on the T cell surface (PD-1, TIM-3, CTLA-4, TIGIT, LAG3, and others) (41); blockade of checkpoint mechanisms has shown clinical benefit in subsets of melanoma and other malignancies (3, 10, 42, 43). Although checkpoint ligand expression (e.g., PD-L1) and neoantigen load clearly contribute (9, 44, 45), no biomarker has emerged that reliably predicts the clinical response to immune checkpoint blockade. We reasoned that single-cell analyses might yield features to elucidate response determinants and possibly identify new immunotherapy targets.

Thus, we analyzed the single-cell expression patterns of 2068 T cells from 15 melanomas. We identified T cells and their main subsets [CD4⁺, regulatory T cells (T_{regs}), and CD8⁺] on the basis of the expression levels of their respective defining surface markers (Fig. 5A, top, and table S12). Within both the CD4⁺ and CD8⁺ populations, a PCA distinguished cell subsets and heterogeneity of activation states on the basis of the expression of naïve and cytotoxic T cell genes (Fig. 5, A and B, and fig. S19).

Next we sought to determine the exhaustion status of each cell from the expression of key coinhibitory receptors (*PDI*, *TIGIT*, *TIM3*, *LAG3*, and *CTLA4*). In several cases, these coinhibitory receptors were coexpressed across individual cells; we validated this phenomenon for PDI and TIM3 by IF staining (Fig. 5C). However, exhaustion gene expression was also highly correlated with the expression of both cytotoxicity markers and overall T cell activation states (Fig. 5B). This observation resembles an activation-dependent exhaustion expression program, such as those reported previously (46, 47). Accordingly, expression of coinhibitory receptors (alone or in combinations) may not be sufficient by itself to characterize the salient functional state of tumor-associated T lymphocytes *in situ* or to distinguish exhaustion from activation.

To define an activation-independent exhaustion program, we leveraged single-cell data from CD8⁺ T cells sequenced in a single tumor (Mel75, 314 cells). These data allowed cytotoxic and exhaustion programs to be deconvolved. Specifically, PCA of Mel75 T cell transcriptomes identified a robust expression module that consisted of all five coinhibitory receptors and other exhaustion-related genes, but not cytotoxicity genes (fig. S21 and table S13).

We used the Mel75 exhaustion program, along with previously published exhaustion programs (46, 48), to estimate the exhaustion state of each cell. An exhaustion state was defined as high or low expression of the exhaustion program relative to that of the cytotoxicity genes (Fig. 5D) (17). Accordingly, we defined exhaustion states in Mel75 and in four additional tumors with the highest number of CD8⁺ T cells (68 to 214 cells per tumor). We identified the top preferentially expressed genes in high-exhaustion cells compared with low-exhaustion cells (both defined relative to the expression of cytotoxicity genes). This allowed us to define a core exhaustion signature across cells from various tumors.

Our core exhaustion signature yielded 28 genes that were consistently up-regulated in high-exhaustion cells of most tumors, including coinhibitory (TIGIT) and costimulatory (TNFRSF9/4-1BB and CD27) receptors (Fig. 5E and table S14). In addition, most genes that were significantly up-regulated in high-exhaustion cells of at least one tumor had distinct associations with exhaustion across the different tumors (Fig. 5F, 272 of 300 genes with $P < 0.001$ by permutation test; fig. S22, A and B; and table S14). These tumor-specific signatures included variable expression of known exhaustion markers (table S14) and

could be linked to immunotherapeutic response or might reflect the effects of previous treatments. For example, CTLA-4 was highly up-regulated in exhausted cells of Mel75 and weakly up-regulated in three other tumors but was completely decoupled from exhaustion in Mel58. Interestingly, Mel58 was derived from a patient with an initial response and subsequent development of resistance to CTLA-4 blockade with ipilimumab (Fig. 5F and fig. S22C). Another variable gene of interest was the transcription factor NFATC1, previously implicated in T cell exhaustion (49). NFATC1 and its target genes were preferentially associated with the activation-independent exhaustion phenotype in Mel75 (fig. S22, D and E), suggesting a potential role of NFATC1 in the underlying variability of exhaustion programs among patients.

Finally, we explored the relationship between T cell states and clonal expansion. T cells that recognize tumor antigens may proliferate to generate discernible clonal subpopulations defined by an identical T cell receptor (TCR) sequence (50). To identify potentially expanded T cell clones, we used RNA-seq reads that map to the TCR to classify single T cells by their isoforms of the V and J segments of the α and β TCR chains, and we searched for enriched combinations of TCR segments. Most observed combinations were found in few cells and were not enriched. However, approximately half of the CD8⁺ T cells in Mel75 had one of the seven enriched combinations identified (FDR = 0.005) and thus may represent expanded T cell clones (Fig. 5G and fig. S23). This putative T cell expansion was also linked to exhaustion (Fig. 5H), such that low-exhaustion T cells were depleted in expanded T cells (TCR clusters with more than six cells) and enriched in nonexpanded T cells (TCR clusters with one to four cells). In particular, the nonexhausted cytotoxic cells are almost all non-expanded cells (Fig. 5H). Overall, this analysis suggests that single-cell RNA-seq may allow for the inference of functionally variable T cell populations that are not detectable with other profiling approaches (fig. S24). This knowledge may empower studies of tumor response and resistance to immune checkpoint inhibitors.

Conclusions

Our analysis has uncovered intra- and interindividual, spatial, functional, and genomic heterogeneity in melanoma cells and associated tumor components that shape the microenvironment, including immune cells, CAFs, and endothelial cells. We identified a cell state in a subpopulation of all melanomas studied that is linked to resistance to targeted therapies, and we used a variety of approaches to validate the presence of a dormant drug-resistant population in a number of melanoma cell lines.

By leveraging single-cell profiles from a few tumors to deconvolve a large collection of bulk profiles from TCGA, we discovered different microenvironments associated with distinct malignant cell profiles. We also detected a subset of genes expressed by one cell type (e.g., CAFs) that

may influence the proportion of other cell types (e.g., T cells); this indicates the importance of intercellular communication for tumor phenotype. Putative interactions between stromal-derived factors and immune cell abundance in melanoma core biopsies suggest that future diagnostic and therapeutic strategies should account for tumor cell composition rather than bulk expression. Furthermore, our data suggest potential biomarkers for distinguishing exhausted and cytotoxic T cells that may aid in selecting patients for immune checkpoint blockade.

Although future work is necessary to clarify the interplay between these cell types and functional states in space and time, the ability to carry out a number of highly multiplexed single-cell observations within a tumor allows us to identify meaningful cell subpopulations and gene expression programs that may inform both the analysis of bulk transcriptional data and precision treatment strategies. Conceivably, single-cell genomic profiling may soon enable a deeper understanding of the complex interplay among cells within the tumor ecosystem and its evolution in response to treatment, thereby providing a versatile new tool for future translational applications.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S24
Table S1 to S16
References (51–67)

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QUANTUM HALL EFFECT

The half-filled Landau level: The case for Dirac composite fermions

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In a two-dimensional electron gas under a strong magnetic field, correlations generate emergent excitations distinct from electrons. It has been predicted that “composite fermions”—bound states of an electron with two magnetic flux quanta—can experience zero net magnetic field and form a Fermi sea. Using infinite-cylinder density matrix renormalization group numerical simulations, we verify the existence of this exotic Fermi sea, but find that the phase exhibits particle-hole symmetry. This is self-consistent only if composite fermions are massless Dirac particles, similar to the surface of a topological insulator. Exploiting this analogy, we observe the suppression of $2k_F$ backscattering, a characteristic of Dirac particles. Thus, the phenomenology of Dirac fermions is also relevant to two-dimensional electron gases in the quantum Hall regime.

Electrons confined to a two-dimensional (2D) plane in a strong magnetic field organize into a plethora of remarkable phases in which correlations play the key role. A global understanding of these phases is provided by topological excitations called composite fermions (CFs) (1, 2). One of the most intriguing phases occurs in the half-filled ($\nu = 1/2$) Landau level, in which CFs were predicted by Halperin, Lee, and Read (HLR) to form a metallic state, a so-called “composite fermion liquid” (CFL) (3). Experiments corroborated this understanding by identifying signs of a Fermi surface despite the intense magnetic field (4–7). Although many studies have produced results in agreement with the HLR theory, one aspect of the $\nu = 1/2$ phase has remained an enigma: a particle-hole (PH) symmetry of the quantum Hall problem projected into the spin-polarized lowest Landau level (LLL) (8–13). The HLR theory attaches fluxes to electrons but not to holes, and because the theory is not confined to the LLL, PH symmetry is broken. One possibility is that PH symmetry is spontaneously broken (14), as is believed to occur in the first excited Landau level (e.g., GaAs at filling $\nu = 5/2$) (15–18), leading to the Moore-Read phase (19).

Recently, theorists have proposed a radical twist to this picture, forging deep connections with the physics of 3D topological insulators (TIs) (20–23). In the new picture (20), CFs are massless Dirac particles, similar to electrons on the surface of a

TI. The CFs are coupled to an emergent gauge field, so the new theory retains the non-Fermi-liquid aspects of HLR; this “Dirac-CFL” is equivalent to the effective field theory for quantum electrodynamics in (2+1) dimensions (QED₃) at finite density. In a TI, the masslessness of the Dirac fermions is protected by the Kramers time-reversal symmetry. Here time-reversal is absent, but the PH symmetry that trades occupied and unoccupied electronic states at $\nu = 1/2$ plays an equivalent role. It is proposed that when PH acts on the CFs, it behaves exactly like Kramers time-reversal symmetry, exchanging states on opposite points of the Fermi surface and forbidding a CF mass term (20). Rather than being an enigma, PH symmetry now plays a starring role.

We address the following two questions. First, is the half-filled Landau level consistent with a composite Fermi liquid, either in its original form or in the Dirac revision? Second, is PH symmetry preserved, and what are the measurable effects if the CFs are indeed Dirac particles? Using large-scale density matrix renormalization group (DMRG) numerical simulations (24), we provide strong evidence for the formation of a CFL in the LLL with realistic Coulomb interactions. We observe a single, nondegenerate Fermi surface consistent with a Luttinger count of CFs, as well as signatures of an emergent gauge field. Furthermore, PH symmetry is preserved in the LLL ground state.

Combining these numerical observations with theoretical insights into the microscopic action of PH yields an argument logically sufficient to conclude that the CF is a Dirac fermion. However, to demonstrate this point directly, we test for the analog of a classic signature of the TI surface: the suppression of $2k_F$ backscattering off impurities that preserve time-reversal symmetry (25, 26). We indeed find that backscattering off PH-symmetric potentials is suppressed in the CFL, reappearing only when PH symmetry-breaking perturbations are introduced. We also find that the effect of small PH symmetry-breaking perturbations present in experiments is very weak, suggesting

that PH symmetry is experimentally relevant at $\nu = 1/2$.

Model and methods

We study electrons on an infinitely long cylinder, in an external magnetic field B , interacting via Coulomb repulsion. There are several advantages to the infinite-cylinder geometry: There are no edge effects, PH symmetry can break spontaneously, and algebraic correlations are possible. For our numerical simulations (27), we truncate the interaction as $V(r) = \exp(-r^2/2\lambda^2)/r$ and project into the LLL. We assume the system is spin-polarized, which occurs in the high-field regime (28). We fix $\lambda = 6\ell_B$, which is large enough to capture the Coulomb interaction physics but small enough to avoid finite-circumference effects. Throughout, the magnetic length $\ell_B \equiv \sqrt{\hbar/(eB)}$ (where $\hbar$ is the Planck constant divided by 2π and e is electron charge) is set to 1; thus, lengths are given in units of ℓ_B and momenta in units of ℓ_B^{-1} . We use the coordinate x along the infinite length and y around the finite circumference L_y of the cylinder.

The states we study are critical and have algebraic correlations along the cylinder, as well as infinite bipartite entanglement. However, infinite DMRG always introduces a finite length scale ξ due to the finite bond dimension χ used in the DMRG variational ansatz. Instead of the usual “finite size scaling,” we perform the so-called “finite entanglement scaling” by running DMRG at various bond dimensions $\chi \approx 10^2$ to 10^4 , and extrapolating the results as $\chi \rightarrow \infty$ (29, 30). This way we can analyze the $\xi \rightarrow \infty$ limit and extract the critical properties of the system on the infinite cylinder.

We describe further details of our DMRG setup in (31). Here, we note that the momentum K_y around the cylinder is conserved in the DMRG, so it must be chosen correctly in order to find the ground state. We find that the momentum K_y of the ground state depends on the circumference L_y . Thanks to inversion symmetry, only two such momenta appear, and we label these momentum sectors “0110” and “0101” (31). Thus, for each circumference, we must run the DMRG twice to determine which sector contains the ground state. Interestingly, these sectors allow us to access different boundary conditions for the CFs.

Mapping the Fermi surface of emergent fermions

In the 2D limit, the CFs are expected to form a circular Fermi surface with Fermi momentum $k_F = 1$ at $\nu = 1/2$, which we can study with our numerics. Although the cylinder is infinite in x , the finite L_y quantizes the momenta k_y into a discrete grid with spacing $2\pi/L_y$. Thus, instead of a circular Fermi surface, we expect a set of “wires” labeled by k_y (Fig. 1A). As L_y is increased, the available k_y s get closer together and the number of wires N_w increases, better approximating the 2D system.

We get a sense of how well we approach the 2D limit in Fig. 2, which shows the electron density-density correlations $D(\mathbf{q}) \equiv \langle \delta\rho_{\mathbf{q}}\delta\rho_{-\mathbf{q}} \rangle$ as

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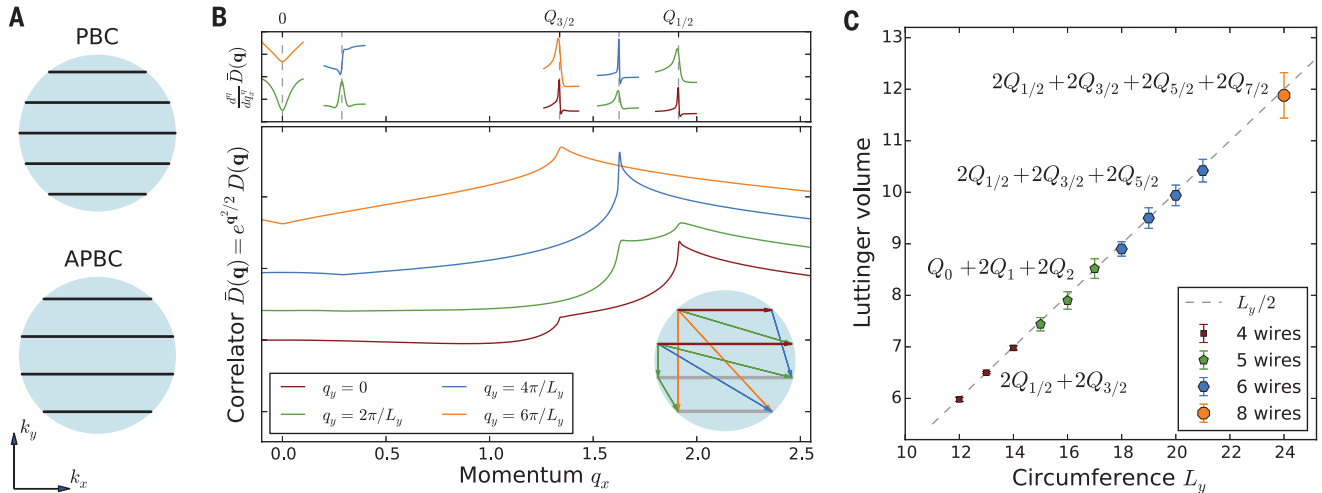


Fig. 1. Mapping the Fermi surface by means of density-density correlations. (A) Boundary conditions on the composite fermions (CFs). Our states can be described by a number of “wires”—slices through the 2D Fermi sea—at various fixed values of k_y . The number of wires N_w is dictated by L_y and the boundary condition of the CFs. An odd N_w corresponds to the periodic boundary condition (PBC); an even N_w corresponds to the antiperiodic boundary condition (APBC). (B) Mapping the Fermi surface via the structure factor. The lower panel shows the “guiding center” density-density correlations, $\exp(\mathbf{q}^2/2) D(\mathbf{q}) = \exp(\mathbf{q}^2/2) \langle \delta \rho_{\mathbf{q}} \delta \rho_{-\mathbf{q}} \rangle$, measured on a cylinder with $L_y = 13$. The singularities arise from CF scattering processes across the Fermi surface. The observed scatterings are illustrated in the inset, with colors corresponding to q_y . The

upper panel shows the derivatives of the correlator, which aid in determining the location of the singularities. (C) Testing Luttinger’s theorem, which states that the area enclosed by the Fermi surface is related to the particle density. On a cylinder, the “area” is given by the sum of the length of each wire in momentum space, which we determined from singularities in plots such as (B). We define Q_m to be the length of the Fermi sea slice at $k_y = (2\pi/L_y)m$ for integer m and plot the resulting sums for various circumferences against the Luttinger prediction. Note that we use the relation $Q_{-m} = Q_m$, a consequence of the 180° rotational symmetry. There is excellent agreement between our data and the Luttinger count. Error bars are estimated on the basis of the finite width of the observed singularities, which is due to our finite correlation length.

a function of momenta $\mathbf{q}$ at $L_y = 24$. [See (31) for details on measuring $D(\mathbf{q})$.] The density structure factor in the 2D limit is expected to be rotationally symmetric with a singularity on a circle of radius $2k_F$. Although we only have access to data at discrete q_y , our measurements still approximate a circular shape, with distinct singular features near $|\mathbf{q}| = 2k_F$. One expects a singularity $D(\mathbf{q}) \sim ||\mathbf{q}| - 2k_F|^\alpha$ with $\alpha = 3/2$ in the CFL for Coulomb electronic interactions, but α is modified for short-range interactions (32–37). Future studies approaching the 2D limit more closely might allow quantitative tests of such predictions (38).

We determine the number and lengths of the CF wires from $D(\mathbf{q})$. The electron density will generically couple to CF scattering processes (with appropriate symmetries):

$$\delta \rho_{\mathbf{q}} = \sum_{\mathbf{k}} A_{\mathbf{k}} \bar{\psi}_{\text{CF};\mathbf{k}} \psi_{\text{CF};\mathbf{k}+\mathbf{q}} + \dots \quad (1)$$

where ψ_{CF} is a composite fermion annihilation operator, $\bar{\psi}_{\text{CF}}$ is the corresponding creation operator, and the ellipsis denotes higher-body processes. A transition of momentum $\mathbf{q}$ across the Fermi surface will contribute a singularity to the structure factor $D(\mathbf{q})$. Because $\mathbf{k}$ and $\mathbf{k} + \mathbf{q}$ are restricted to the wires, we can use the singularities in $D(\mathbf{q})$ to determine the configuration of the wires. For example, the singularities in $D(q_x, q_y = 0)$ contain transitions within the wires (i.e., fixed k_y) and reveal the lengths of the wires inside the Fermi sea. At other values of q_y , the singularities correspond to processes that connect the ends of the wires whose k_y momenta differ by q_y .

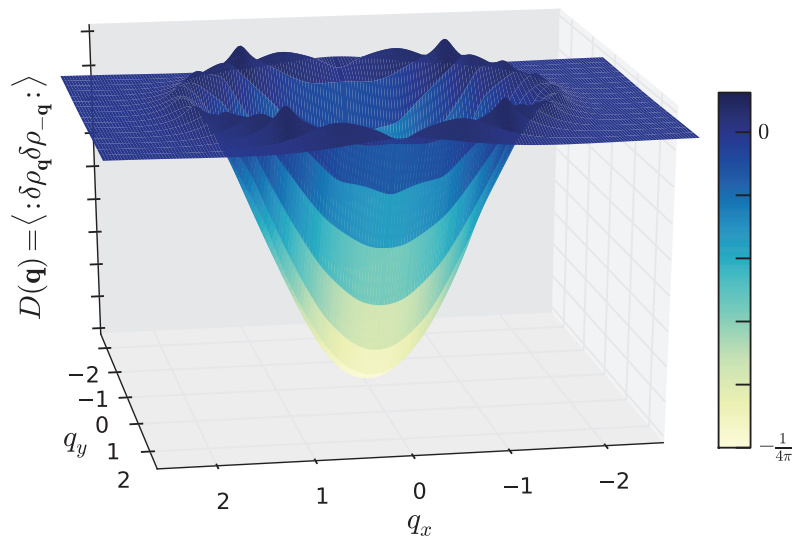


Fig. 2. Two-dimensional momentum space view of density-density correlations. $D(\mathbf{q}) = \langle \delta \rho_{\mathbf{q}} \delta \rho_{-\mathbf{q}} \rangle$ is plotted as a function of 2D momenta $\mathbf{q} = (q_x, q_y)$. The cylinder circumference is $L_y = 24$, where we see eight slices through the Fermi sea. Descendants of a singular $2k_F$ circle in $D(\mathbf{q})$ appear in cuts at q_y between $-7(2\pi/L_y)$ and $7(2\pi/L_y)$. At this large circumference, the correlations approach those of a 2D system.

Slices of the density structure factor for $L_y = 13$ are shown in the lower panel of Fig. 1B. Note that here we plot the “guiding center” structure factor $\bar{D}(\mathbf{q}) = \exp(\mathbf{q}^2/2) D(\mathbf{q})$, which has the same singularities as $D(\mathbf{q})$ but is more conveniently scaled (31). Visual inspection reveals some of the expected singularities, and we can increase the contrast by taking a “fractional” derivative with respect to q_x (39). We calculate an n th derivative by multiplying

the real-space correlations by $|x - x'|^n$ before Fourier transformation. In the upper panel of Fig. 1B, we show the results for various $n \in (0.5, 1.5)$, with n chosen for each singularity individually. This method reveals many singularities that are barely visible in the raw data.

Although the physical electrons have periodic boundary conditions, the CFs need not have them. An arbitrary flux Φ_{int} of the emergent internal

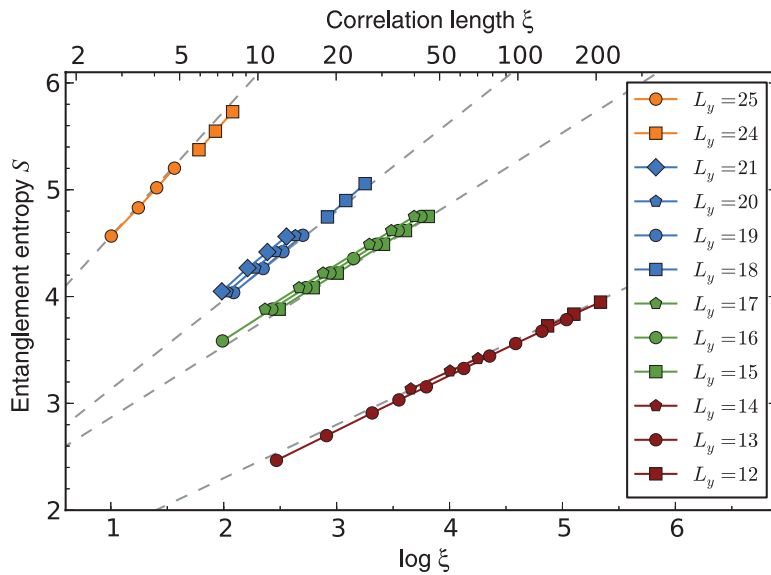


Fig. 3. Entanglement entropy versus correlation length. Data are taken at a variety of circumferences L_y ; different data points at the same size correspond to different bond dimensions $\chi = 600$ to 12,000. Both S and ξ increase as χ is increased. For a quasi-1D critical system, we expect a linear relationship between S and $\log \xi$ with the slope proportional to the central charge c (see Eq. 3). The dashed lines (from bottom to top) correspond to $c = 3, 4, 5$, and 7 .

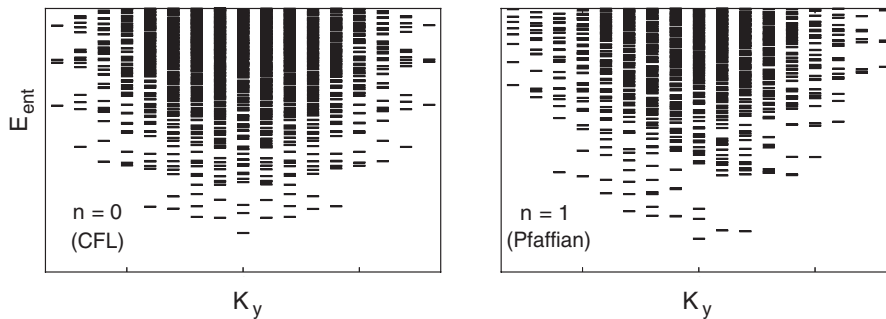


Fig. 4. Contrasting orbital entanglement spectra of the $n = 0$ and $n = 1$ Landau levels at half-filling. These results correspond to experiments at $\nu = 1/2$ and $\nu = 5/2$, respectively. Data are at $L_y = 19$; $n = 0$ is the gapless CFL phase, and $n = 1$ is consistent with the gapped Pfaffian phase. E_{ent} corresponds to the eigenvalues of the reduced density matrix for the left half of the cylinder. Each eigenvalue is associated with a momentum around the cylinder K_y , just like an energy spectrum. PH acts as a reflection $K_y \leftrightarrow -K_y$. The characteristic chiral “dispersion” at $n = 1$ clearly breaks PH, whereas $n = 0$ is PH-symmetric.

gauge field can thread the cylinder; quantizing the CF momenta as

$$k_y \in \frac{2\pi}{L_y} \left(n + \frac{\Phi_{\text{int}}}{2\pi} \right) \quad (2)$$

(40), where n is an integer. Being a dynamical degree of freedom, the emergent flux Φ_{int} will adjust to lower the energy of the system, depending on L_y . Only two cases respect the 180° rotational symmetry: the periodic boundary condition (PBC) with $\Phi_{\text{int}} = 0$, and the antiperiodic boundary condition (APBC) with $\Phi_{\text{int}} = \pi$. As shown in Fig. 1A, the boundary condition dictates the parity of the number of wires: PBC yields an odd N_w , whereas APBC yields an even N_w .

The observed singularities at $L_y = 13$ are in good agreement with an $N_w = 4$ state with APBC.

The arrows in the inset of Fig. 1B show the momenta at which singularities are expected; all of these are observed in the main plot. We obtain similar data for other circumferences L_y (31), with Fermi surfaces cut by $N_w = 4, 5, 6$, and 8 wires (41). We find that the parity of N_w (and hence the boundary condition) depends only on the momentum sector used to initialize DMRG, such that 0101 always yields an odd N_w , whereas 0110 yields an even N_w . This correspondence between the momentum sector and the boundary condition is consistent with the CF theory, where it arises from an anomaly in finite-density QED₃ (31).

A useful check on our conclusions so far is a comparison to Luttinger’s theorem (42) for the CFs. The electron density per unit length of the cylinder is $\rho_{1D} = L_y(v/2\pi\ell_B^2)$. The Luttinger count

requires that $2\pi\rho_{1D}$ be equal to the volume of the Fermi sea (which here is the sum of the lengths of the wires), as predicted by both the HLR and Dirac-CFL theories at $v = 1/2$. We find excellent agreement with this prediction in Fig. 1C. Our findings are in contrast to a recent suggestion (43) that the Luttinger count is violated by the CF model wave functions.

We can learn more about the CFs by measuring the central charge c , which for our purposes can be thought of as the number of gapless modes in our quasi-1D system. This is determined from the scaling between the entanglement entropy S and the DMRG ansatz correlation length ξ as the bond dimension χ is increased. Whereas in a gapped state S and ξ converge with χ , for a critical state both quantities diverge. The central charge is extracted using the relation

$$S = \frac{c}{6} \log \xi + \text{const.} \quad (3)$$

(44). Figure 3 shows S versus $\log \xi$ as χ is varied. The dashed lines correspond to $c = 3, 4, 5$, and 7 . Clearly c increases with L_y , with each new wire adding 1 to the central charge. The preceding analysis found $N_w = 4, 5, 6$, and 8 for these systems, respectively, which allows us to deduce the relation $c = N_w - 1$. Our data rule out an ordinary Fermi liquid, for which $c = N_w$. Instead, they confirm the emergence of a gauge field in the CFL; in the quasi-1D limit, the effect of the gauge field is to gap out the total gauge charge mode, reducing c by 1 (31).

Particle-hole symmetry and the absence of $2k_F$ backscattering

Our numerical findings thus far are expected of both the HLR and Dirac-CFL phases; to distinguish them, we turn to PH symmetry. The symmetry is generated by $\mathcal{PH}$, an anti-unitary operator that swaps creation operators $c^\dagger$ with annihilation operators c :

$$\mathcal{PH}: c_j \leftrightarrow c_j^\dagger, i \leftrightarrow -i, |000\cdots\rangle \rightarrow |111\cdots\rangle \quad (4)$$

PH symmetry is an exact symmetry of the Hamiltonian when the interaction is projected into the LLL, which is justified when the cyclotron energy $\hbar\omega_c \rightarrow \infty$. In reality, PH symmetry is weakly broken by the finite ratio of the Coulomb energy to the cyclotron energy. [See (31) for quantitative evidence that this breaking is weak in typical GaAs samples.]

The HLR construction breaks PH symmetry, whereas the Dirac-CFL explicitly preserves PH symmetry. Our first test is for spontaneous PH symmetry breaking, which can happen in our infinite-cylinder numerics, as PH is a discrete symmetry that we do not explicitly enforce. Indeed, our numerical DMRG runs clearly break PH symmetry at $v = 5/2$, randomly selecting either the Pfaffian or anti-Pfaffian state (27). Furthermore, our numerics will never produce a symmetric superposition (“cat state”) of the HLR and its PH conjugate (31).

To test for spontaneous symmetry breaking, we compute the overlap between the ground state and its PH conjugate,

$$\langle \Psi | \mathcal{PH} | \Psi \rangle = (1 - \epsilon)^{N_{\text{orb}}} \quad (5)$$

which should decrease exponentially with the number of orbitals (i.e., system size) N_{orb} . DMRG directly computes the overlap per orbital $1 - \epsilon$; $\epsilon > 0$ indicates symmetry breaking. For comparison, we calculate the same overlap in the $n = 1$ Landau level (i.e., $\nu = 5/2$ mentioned above) (17). As an example, at $L_y = 16$ for $\nu = 1/2$, we find $\epsilon < 6 \times 10^{-5}$, whereas for $\nu = 5/2$ we find $\epsilon \approx 0.022 \pm 0.002$. At $\nu = 5/2$, this implies that the total overlap on a 16×16 torus with $N_{\text{orb}} \approx 16^2/2\pi$ is $0.978^{N_{\text{orb}}} \approx 0.4$, so the PH symmetry breaking is fairly strong. The difference between $\nu = 1/2$ and $\nu = 5/2$ is also manifest in qualitatively different “entanglement spectra” (45), as illustrated in Fig. 4. Similar results hold at $L_y = 13$ and 18. Therefore, barring some transition at even larger L_y , we conclude that the $\nu = 1/2$ CFL state is PH-symmetric.

Given the presence of PH symmetry, we can ask how the CFs transform under this symmetry. According to (20–22), PH acts like time-reversal symmetry on the CFs, implying a two-fold Kramers degeneracy whenever an odd number of CFs is present. Indeed, this feature is already apparent by the very definition of $\mathcal{PH}$, because a system with N_{orb} orbitals transforms as

$$\mathcal{PH}^2 = (-1)^{N_{\text{orb}}/2} \quad (\text{with } N_{\text{orb}} \text{ even}) \quad (6)$$

(31, 46). Thus, at filling $\nu = 1/2$, adding a pair of fluxes plus an electron—that is, a CF—changes $\mathcal{PH}^2$ by -1 .

The Kramers degeneracy of the CFs leads to their two-fold “pseudospin” degrees of freedom, which are exchanged via PH symmetry. Two kinds of low-energy gapless theories are possible. The first is a pair of CF Fermi surfaces that are exchanged by PH symmetry (or possibly are Rashba split). The other is a single nondegenerate Fermi surface in which the CF pseudospin is locked to the momentum—that is, a Dirac cone. Our numerical finding of a single Fermi surface with the correct Luttinger volume implies that the latter proposal is realized, consistent with (20–22).

One of the most striking consequences of a Dirac cone is that CFs accumulate a π Berry phase when circling the Dirac point. In topological insulators, this Berry phase prevents precise $2k_F$ backscattering off time-reversal-symmetric impurities on the surface. If our system is a Dirac CFL, we should observe the same effect, with $\mathcal{PH}$ playing the role of time-reversal operator. At the level of equal-time correlation functions, we expect that a Hermitian, PH-even operator P will not have strong singularities in $\langle P_{\mathbf{q}} P_{-\mathbf{q}} \rangle$ at $|\mathbf{q}| = 2k_F$ in two dimensions (31). The electron density studied earlier is odd under PH, so it does not have this property. A candidate PH-even observable is $P(\mathbf{r}) \equiv \delta \rho(\mathbf{r}) \nabla^2 \rho(\mathbf{r})$. A contribution to this correlation function (31) is shown in Fig. 5, measured for the same four-wire APBC CFL as in Fig. 1B. The measurements are done at $q_y = 2\pi/L_y$, so as to

probe the CF scattering from the right Fermi point at $k_y = \pi/L_y$ to the left point at $k_y = -\pi/L_y$, which corresponds to exact $2k_F$ backscattering in two dimensions.

Despite showing many other singularities, all of which can be accounted for by various multiple-CF scattering processes (31), the result is perfectly smooth in the vicinity of the (already measured) momentum corresponding to this exact $2k_F$ backscattering. We also confirm the absence of the exact backscattering in the PH-symmetric model for a wider cylinder with $L_y = 16$, which realizes a five-wire CFL (31).

If the absence of the $2k_F$ backscattering is truly due to the PH symmetry rather than to some peculiarity of P , we expect that the backscattering should return if PH symmetry is explicitly broken. We break PH symmetry by adding a second quantum well at a distance l_B above the first, with Coulomb intra- and interwell interactions. The second well has a chemical potential μ relative to the first one, and electrons can tunnel between the wells. When $\mu = \infty$, electrons remain in the first well, and we recover PH symmetry. As μ decreases, electrons tunnel to the second well, breaking PH symmetry, which should induce backscattering.

Figure 5 shows data confirming this hypothesis for $L_y = 13$. The tunneling strength is fixed at $t = 0.01$ in units of $e^2/(4\pi l_B)$. The system remains in an effectively one-well CFL phase for $\mu > 0.05$ [for smaller values of μ , the system becomes a two-component Halperin (331) state (47)]. As μ is decreased within the CFL phase, the $2k_F$ singularity reappears. Note that the measured central charge of the CFL phase remains unchanged;

thus, the gapless Fermi surface is stable against PH symmetry-breaking perturbations (31).

Our findings appear to violate the famous 2D fermion doubling theorem: A single time-reversal-symmetric Dirac cone is anomalous, so it cannot be realized in two dimensions. However, as noted in (22), our Dirac CF is coupled to an emergent gauge field with unusual compactification condition, which “cures” the anomaly. The half-filled LLL has yet another anomalous property. Just as the fully symmetric surface of a topological insulator must be nontrivial—that is, either gapless or topologically ordered—a PH-symmetric state of the half-filled Landau level must be nontrivial. Indeed, it must have a Hall conductance of $\sigma^{xy} = e^2/2h$, and if the state is gapped, this requires fractionally charged excitations and hence topological order. In fact, the microscopic PH behaves exactly like time-reversal symmetry on the surface of a 3D topological phase [in class AIII (31)]. How does this occur in a purely 2D setting? For any symmetry that is locally implemented, one can always obtain a symmetric and trivial product state. The key observation resolving this apparent paradox is that PH symmetry of the LLL is special, in that its action is nonlocal. The nonlocality is ultimately tied to the fact that the Landau level orbitals $\phi_j(\mathbf{r})$ cannot be localized because of the topological nature of the LLL.

Discussion

A number of open questions of relevance to both experiment and theory remain. First, given the experimental success in observing the phenomenology of a Dirac cone in TI surfaces and a CF

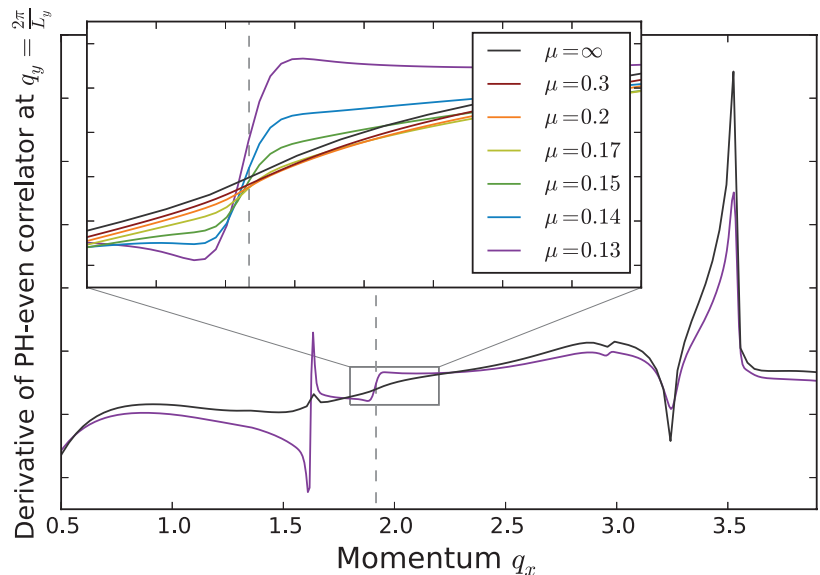


Fig. 5. The extinction of $2k_F$ backscattering off PH-symmetric impurities. A QH bilayer with chemical potential imbalance μ allows us to continuously tune from a PH-symmetric model ($\mu = \infty$) to a PH-broken one (μ finite). We compute the correlation function of a PH-even operator $\langle P_{\mathbf{q}} P_{-\mathbf{q}} \rangle$ for $q_y = 2\pi/L_y$ at $L_y = 13$ and plot its derivative with respect to q_x to bring out singularities. For the PH-symmetric case, there are many singularities, but noticeably absent is any kink at $|\mathbf{q}| = 2k_F$ (marked by a dashed line). This demonstrates the Dirac nature of the CFs: P is even under PH, whereas scattering a CF across the Fermi surface to its antipode is PH-odd in the Dirac theory. At finite chemical potential μ , the bilayer setup explicitly breaks PH symmetry, and a kink at $2k_F$ continuously reappears.

Fermi surface in GaAs, it would be extremely interesting to find experimental probes of PH symmetry and the potential Dirac nature of CFs. Existing experiments are already of some relevance, such as recent measurements (48) of CF “geometric resonances” induced by small deviations of B away from half-filling. DMRG could be of use in guiding and interpreting such experiments—for instance, by computing static structure factors, impurity responses, and the behavior of the CFL at $\nu = 1/2 + \delta$.

Second, a PH-symmetric version of a paired phase, the “PH-Pfaffian” (20), has been proposed as a gapped topological state at $\nu = 1/2$; this phase had previously been proposed as the surface topological order of a PH-symmetric (class AIII) 3D topological superconductor (49). Although our results appear to rule out this possibility in the $n = 1$ LL of GaAs, it would be interesting to search for such a phase in broader phase diagrams of two-dimensional electron gases.

Finally, similar theories with a surface of emergent gapless fermions coupled to an emergent gauge field arise for other exotic phases with itinerant fractionalized excitations, such as spin liquids with a spinon Fermi sea, Bose metals, and electron non-Fermi-liquid metals. Much recent theoretical effort has aimed to clarify the status of such field theories (37, 50–52), although it remains not fully settled away from artificially controlled limits. Unbiased numerical studies of the CFL thus bear directly on open questions for all these other non-Fermi liquids. Thanks to many innovations in the DMRG for the fractional quantum Hall effect, the present CFL work goes to effectively much wider strips and is much closer to the 2D physics than earlier studies. It would be useful to push the numerical CFL study even closer to 2D and to develop scaling analysis tools for addressing 2D questions, such as detailed characterization of the $2k_F$ singularity in the structure factor.

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- We do not have data for every system size for a given boundary condition because the simulations fail to converge when a wire is close to the edge of the Fermi sea. The different “root configurations” 0110 and 0101 (i.e., K_y momentum sectors) give different CF boundary conditions and allow us to access different sizes: The wires for one boundary condition are closest to the edge of the Fermi sea at the sizes when the

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/352/6282/197/suppl/DC1
Supplementary Text
Figs. S1 to S7
References (54–62)

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REPORTS

QUANTUM SIMULATION

Extended Bose-Hubbard models with ultracold magnetic atoms

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The Hubbard model underlies our understanding of strongly correlated materials. Whereas its standard form only comprises interactions between particles at the same lattice site, extending it to encompass long-range interactions is predicted to profoundly alter the quantum behavior of the system. We realize the extended Bose-Hubbard model for an ultracold gas of strongly magnetic erbium atoms in a three-dimensional optical lattice. Controlling the orientation of the atomic dipoles, we reveal the anisotropic character of the onsite interaction and hopping dynamics and their influence on the superfluid-to-Mott insulator quantum phase transition. Moreover, we observe nearest-neighbor interactions, a genuine consequence of the long-range nature of dipolar interactions. Our results lay the groundwork for future studies of exotic many-body quantum phases.

Dipolar interactions, reflecting the forces between a pair of magnetic or electric dipoles, account for many physically and biologically important phenomena. These range from quantum many-body phases (1, 2) to

liquid crystals and ferrofluids (3, 4) to the mechanisms underlying protein folding (5). The distinguishing feature of the dipole-dipole interaction (DDI) is its long-range and anisotropic character (6): Two dipoles oriented in parallel repel each

other, whereas the interaction between two head-to-tail dipoles is attractive. Notable progress toward the ability to study DDI has been made recently in systems containing electric dipoles, such as gases of polar molecules (7) and Rydberg ensembles (8); similarly, the recent experimental advances in creating quantum degenerate gases of bosonic and fermionic magnetic atoms, including Cr (9–11) and the lanthanides Er (12, 13) and Dy (14, 15), have now opened the door to a study of magnetic dipolar interactions.

Ultracold lanthanide atoms have an open electronic f shell and anisotropic interactions; they are characterized by unconventional low-energy scattering properties, including the proliferation of Feshbach resonances (16). This complexity manifests itself in quantum many-body dynamics: By preparing quantum degenerate lanthanide gases in optical lattices, we can realize extended Hubbard models for bosonic and fermionic atoms (2, 17). In addition to the familiar single-particle tunneling and isotropic onsite interactions (as for contact interactions in alkali), dipolar interactions give rise to anisotropic onsite interactions and density-induced tunneling (DIT) and activate nearest-neighbor (offsite) interactions (NNI). Such extended Hubbard models have been extensively studied in theoretical condensed matter physics and quantum materials science (18, 19), and it is the competition between these unconventional Hubbard interactions that underlies the prediction of exotic quantum phases such as supersolid, stripe, and checkerboard phases (17, 20–26).

In our experiment, an ultracold dipolar gas of ^{168}Er atoms is prepared in a three-dimensional (3D) optical lattice. The atoms are fully spin-polarized in their lowest Zeeman sublevel (12) and feature a magnetic moment μ of 7 Bohr magneton. The experiment starts by adiabatically loading a Bose-Einstein condensate (BEC) of about 1.5×10^5 atoms from an optical dipole trap (ODT) into a 3D optical lattice. The lattice is created by two retroreflected 532-nm laser beams, defining the horizontal xy plane, and one 1064-nm beam, nearly collinear with the vertical (z) direction, defined by gravity (Fig. 1A) (27). The lattice has a cuboid unit cell with lattice constants $d_{x,y} = 266$ nm and $d_z = 532$ nm, which for ^{168}Er correspond to the recoil energies $E_{R,x} = E_{R,y} = \hbar \times 4.2$ kHz and $E_{R,z} = \hbar \times 1.05$ kHz, $\hbar$ being Planck's constant. The lattice can be controlled by independently changing the depths associated with the lattice beams in each direction, (s_x, s_y, s_z) , measured here in units of the corresponding recoil energies. The dipole orientation, quantified by the polar angles θ and ϕ (Fig. 1A, inset), is varied by changing the direction of the polarizing magnetic field (27).

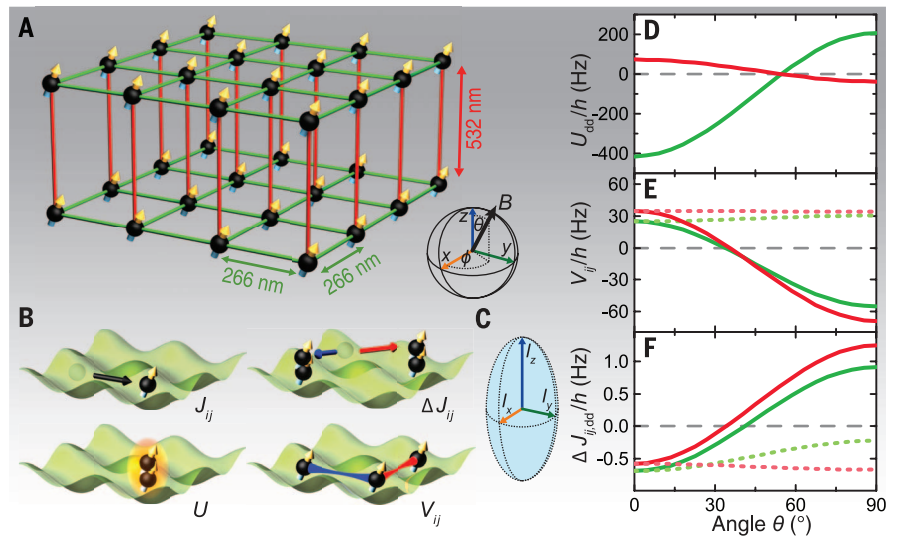


Fig. 1. Magnetic dipoles in a 3D optical lattice. (A) Schematic of our lattice geometry, where the lattice spacings are indicated. The dipole orientation, given by the polarizing magnetic field B , is quantified by the polar angles θ and ϕ with respect to our coordinate system. (B) Illustration of the contributing terms in the eBH model: tunneling matrix element J_{ij} , DIT matrix elements ΔJ_{ij} , onsite interaction U , and NNI V_{ij} . (C) Illustration of the single-site density distribution, where the harmonic oscillator lengths are indicated. (D to F) Calculated values of the DDI-dependent terms as a function of θ for $\phi = 0^\circ$ in the MI phase, $(s_x, s_y, s_z) = (15, 15, s_z)$ with the s_z set by the AR for the cases AR = 1 (red) and AR = 2 (green). (D) shows U_{dd} . (E) and (F) show V_{ij} and $\Delta J_{ij,dd}$, respectively, for bond and hopping directions ij , both along x (solid lines) and y (dotted lines). The dashed lines indicate the case without DDI. U_s and J_{ij} are independent of θ , and their values for the two configurations considered are $U_s = 3749$ Hz (1775 Hz) for AR = 1 (2) and $J_{ij} = 27$ Hz for $ij = x$ or y .

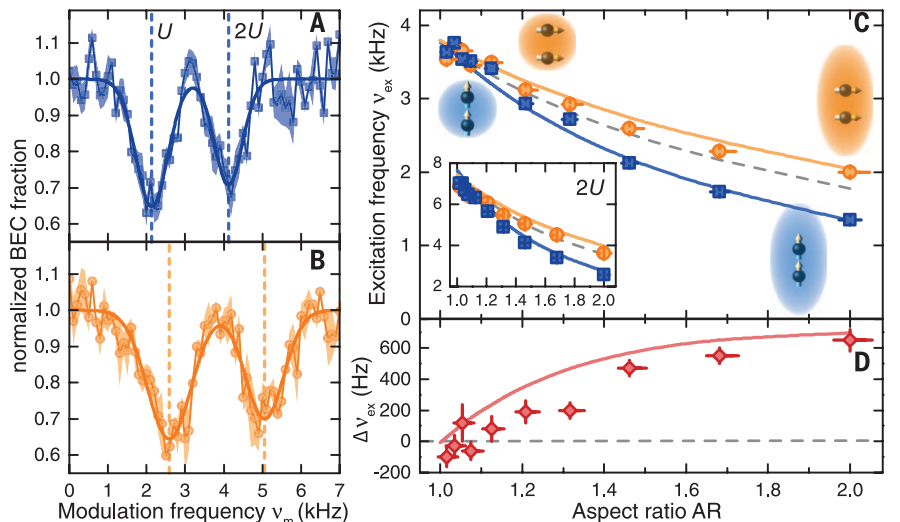


Fig. 2. Measurement of the onsite interactions. (A and B) Excitation spectrum of the MI state for dipole orientations $\theta = 0^\circ$ (A) and $\theta = 90^\circ$ (B) with $\phi = 0^\circ$. The modulation spectroscopy is performed along the x axis at $(s_x, s_y, s_z) = (15, 15, 52.5)$, corresponding to $AR \approx 1.46$, and the remaining BEC fraction is measured after adiabatically ramping down the lattice depths to zero. From a double Gaussian fit to the data (solid line), we extract the resonant excitation frequency ν_{ex} for the U and $2U$ feature. (C) ν_{ex} for the loss feature at U and $2U$ (inset) as a function of AR for $\theta = 0^\circ$ (squares) and $\theta = 90^\circ$ (circles). (D) Difference in excitation frequencies ν_{ex} relative to the two dipole orientations, $\Delta\nu_{ex}$, as a function of AR. The error bars for all figures are the sum of the SEM and systematic errors (27). The theoretical model [solid lines in (C) and (D)] also includes the effect of the NNI, which shifts the excitation frequency by up to 3% (see Fig. 3). Dashed lines: calculations accounting only for the isotropic (contact) interaction.

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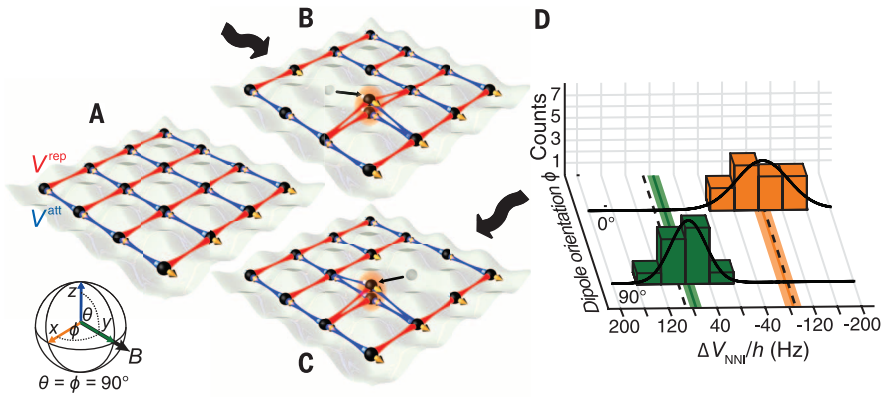


Fig. 3. Nearest-neighbor interactions. (A) Initial system in the MI regime with dipole orientation $\theta = 90^\circ$, $\phi = 90^\circ$. Driving excitations along y (B) or x (C) leads to two different particle-hole energy gaps, $U - V^{\text{att}}$ and $U - V^{\text{rep}}$, respectively. Here, $V^{\text{att}} = V_{ij}$, with in-plane head-to-tail dipole orientations and $V^{\text{rep}} = V_j$ for the in-plane side-by-side orientation. The difference between the two resonant energies ΔV_{NNI} , equal to $-V^{\text{att}} + V^{\text{rep}}$, reveals the NNI. (D) Histogram of our measurements of ΔV_{NNI} for two dipole orientations, $\phi = 0^\circ$ and $\phi = 90^\circ$. The black solid curves are the normal distributions of the corresponding data. The two dashed lines show the theoretical expectation values, whereas the orange and green lines show the corresponding measured values, with the shaded areas indicating the SEM.

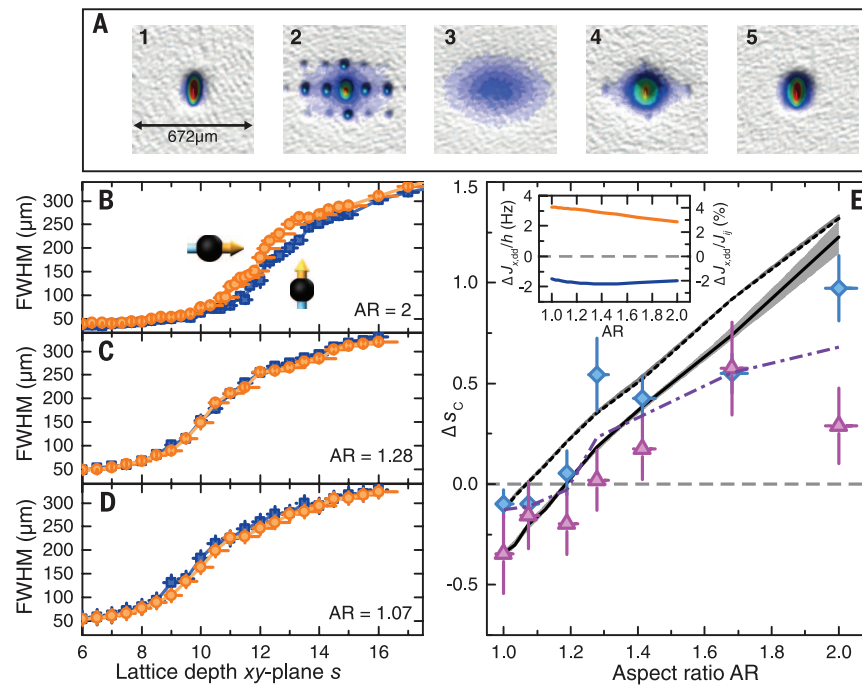


Fig. 4. SF-to-MI transition. (A) Time-of-flight absorption images of the atomic cloud at $\theta = \phi = 0^\circ$, taken 27 ms after a sudden release from the 3D lattice with $s_{x,y,z} = s$, where $s = 0$, $s = 10$, $s = 22$ for (A), 1 to 3, respectively, and $s = 4$, $s = 0$ for (A), 4 to 5, respectively. The distribution of the interference peaks (A2) reflects the anisotropy of the lattice in combination with the orientation of the reciprocal lattice with respect to the imaging light (27). (B to D) The FWHM of the central interference peak as a function of the lattice depth in the xy plane for $\text{AR} = 2$; 1.28; 1.07, with dipoles oriented along $\theta = 0^\circ$ (blue squares) and $\theta = 90^\circ$ (orange circles) with $\phi = 0^\circ$. (E) Δs_c as a function of the AR. We extract $s_c(\theta)$ using both a double-line fit (triangles) and the visibility method (diamonds) (27). The error bars for Δs_c represent the SEM from the fits, and the dot-dashed line is the weighted mean of the two methods. We compare our data with mean-field calculations in the presence (solid line) and absence (dotted line) of the DIT. The gray shaded regions show the SEM, resulting from the resolution of the calculation (27). The dashed line represents the purely contact-interacting case. The inset shows the calculated values of the DIT along the x direction, $\Delta J_{x,\text{dd}}/h$, for $\theta = 0^\circ$ (blue) and $\theta = 90^\circ$ (orange), with $\phi = 0^\circ$ and $s_{x,y} = 10$, which is close to the phase transition point, in units of frequency (left axis) and in units of the single-particle tunneling $J_{ij} = 80.5$ Hz in the xy plane (right axis).

When increasing the lattice depths, we can prepare the Er atoms in the Mott insulator (MI) state by driving the superfluid (SF)-to-MI phase transition, as described below.

The dynamics of Er atoms in the optical lattice are described by an extended Bose-Hubbard (eBH) model with Hamiltonian (17, 28)

$$H = -\sum_{\langle ij \rangle} \left[(J_{ij} + \Delta J_{ij}(n_i + n_j - 1)) b_i^\dagger b_j + \text{h.c.} \right] + \frac{U}{2} \sum_i n_i(n_i - 1) + \sum_{\langle ij \rangle} V_{ij} n_i n_j \quad (1)$$

Here, $b_i^\dagger$ (b_i) are the bosonic creation (annihilation) operators of an atom at site i , $n_i = b_i^\dagger b_i$ is the associated number operator, and $\langle ij \rangle$ denotes pairs of adjacent sites. All terms of the eBH are illustrated in Fig. 1B. The interactions manifest themselves in both the tunneling dynamics, the onsite (U) interaction, and the offsite (V_{ij} , approximated to NNI) interaction. In addition to the single-particle hopping, which here has amplitudes J_{ij} reflecting the anisotropy of the optical lattice, Eq. 1 includes DIT terms (ΔJ_{ij}). The onsite interaction (U) and DIT terms (ΔJ_{ij}) have contributions (U_s and $\Delta J_{ij,s}$) from the short-range contact interaction, which is proportional to the s -wave scattering length a_s ; they also have contributions (U_{dd} and $\Delta J_{ij,\text{dd}}$) from the long-range DDI, which is proportional to μ^2 (17, 27). The DIT has recently been observed in purely contact-interacting systems (29, 30). The NNI (V_{ij}) is a term that genuinely originates from the long-range DDI. The NNI in spin-polarized dipolar systems is qualitatively different from the Heisenberg spin-spin interaction between atoms at neighboring sites $\langle ij \rangle$ (31, 32), which arises from super-exchange processes in Hubbard dynamics in second-order virtual hopping processes ($\sim J_{ij}^2/U$) in the limit of large onsite interaction, and different from dipolar spin-exchange interactions (7) and full Heisenberg-type interactions (10), driving flip-flop dynamics.

The angle dependence of the DDI reveals itself more prominently in combination with anisotropic geometries (33). By changing the lattice depths (s_x, s_y, s_z) in the three directions independently, we can control the aspect ratio (AR) of the 3D density distribution at a given lattice site (Fig. 1C)—i.e., we can shape the anisotropy of the 3D Wannier function. For simplicity, we define the AR using the harmonic approximation, $\text{AR} = l_z/l_{x,y}$, where l_z ($l_x = l_y$) is the harmonic oscillator length along the z (xy) direction of the local atomic well. In our experiment $s_x = s_y$, such that z is the anisotropy axis. The symmetric condition, defined as $U_{\text{dd}} = 0$, is slightly shifted with respect to $\text{AR} = 1$, when using Wannier functions (e.g., $\text{AR} = 1.05$ for $s_x = s_y = 15$ and $\text{AR} = 1.07$ for $s_x = s_y = 10$) (34). For U_{dd} , the relative weight between the attractive and repulsive contributions can be tuned by changing the dipole orientation relative to the anisotropy axis of the onsite density distribution and the AR (Fig. 1D). In contrast, the NNI V_{ij} is controlled mainly through the orientation of the dipoles with respect to the bond direction ij (Fig.

1E). Finally, $\Delta J_{ij,dd}$ depends both on the orientation of the dipoles relative to the bond direction and the anisotropy axis and on the AR (Fig. 1F).

We first investigated the effect of the DDI on the onsite interaction (U_{dd}) by performing spectroscopic measurements. We prepare our system deep in the MI phase and probe the energy gap in the excitation spectrum for different dipole orientations. This energy gap, associated with particle-hole excitations, is U for atoms in singly or doubly occupied Mott shells and $2U$ at the border between the two shells and at doublon defects within the first Mott shell (35–38). We excite the MI state by applying a sinusoidal modulation of frequency ν_{ex} on the intensity of the x -lattice beam. When $h\nu_{ex}$ matches U or $2U$, we observe a resonant depletion of the remaining condensate fraction after ramping down the lattice (27). We perform the measurement for $\theta = 0^\circ$ and $\theta = 90^\circ$ (Fig. 2, A and B) with $\phi = 0^\circ$ and observe that the resonance positions clearly depend on the dipole orientation, consistent with our expectation. To further explore this effect, we repeat the measurement for different values of the AR (Fig. 2C). At the symmetric condition (AR = 1.05), we observe that the excitation gap loses its angle dependence, showing that U_{dd} averages to zero (39). As the spatial distribution is deformed toward larger ARs, we find a clear deviation from the purely contact-interaction case (dashed lines), with a smaller energy gap for dipoles at $\theta = 0^\circ$ and a larger one for dipoles at $\theta = 90^\circ$. For large ARs, the dipole orientation substantially affects the incompressibility of the corresponding Mott state, with the energy difference between the two dipole configurations of up to $h \times \Delta\nu_{ex} = h \times 600$ Hz (Fig. 2D). The observed angle dependence is well described within our eBH model (Fig. 2, C and D, solid lines). The theory for Fig. 2D is parameter free, whereas for Fig. 2C, a_s is the only fit parameter. From the fit we find $a_s = 137(1)a_0$, with a_0 being the Bohr radius, which is consistent with previous measurements based on thermalization experiments (12). Our measurement shows that U_{dd} plays a fundamental role in the stability of the MI phase: It can either protect the MI phase for the dominantly repulsive DDI ($\theta = 90^\circ$) or make it more susceptible to excitations for the dominantly attractive case ($\theta = 0^\circ$).

The energy gap in the MI phase also depends on the NNI between atoms occupying adjacent lattice sites. To isolate the contribution of the NNI in the eBH, we designed a dedicated measurement scheme based on modulation spectroscopy in the 2D short-spacing lattice plane (xy plane), where the NNI is stronger (Fig. 3A). For a system with only onsite interactions, the energy gap associated with the particle-hole excitation does not depend on the direction of excitation—i.e., on the direction of the modulated beam. In contrast, a system including anisotropic NNI will exhibit a modification of the energy gap according to the excitation direction as the energy gap equals $U - V_{ij}$ for excitations along the bond direction ij . Hence, the difference between the two resonance frequencies measured by modulating s_x and s_y , denoted as $\Delta V_{NNI}/h$, directly reveals the existence of the NNI

as the onsite contribution cancels out. Our scheme is illustrated in Fig. 3, A to C, for the case $\theta = 90^\circ$, $\phi = 90^\circ$. Here, one bond of attractive (repulsive) NNI with energy V^{att} (V^{rep}) is destroyed during the excitation along (perpendicular to) the dipole orientation such that $\Delta V_{NNI} = -V^{\text{att}} + V^{\text{rep}}$. Our measurement for two dipole orientations in the plane with $\phi = 90^\circ$ (0°) gives $\Delta V_{NNI}/h = +74(10)$ Hz [$-87(14)$ Hz]. Notably, $|\Delta V_{NNI}|$ is similar for both values of ϕ , as expected from the symmetry between these two configurations, and is close to the theoretical expectation $h \times 91$ Hz (27), as shown in Fig. 3D.

Finally, we use our understanding of the angle dependence of the Hamiltonian to modify the many-body phase transition from a SF to a MI state by changing θ and AR. Figure 4A shows the textbook signature of the phase transition in the momentum distribution of a lattice-confined gas (40): The interference pattern of the SF phase progressively disappears as the system is driven into the MI phase by increasing the lattice depths. However, in stark contrast with the purely contact-interaction case as in previous alkali experiments, we observe that the dynamics of the phase transition has a clear angle dependence. The full width at half maximum (FWHM) of the central interference peak reveals a different evolution with lattice depths for $\theta = 90^\circ$ as compared with $\theta = 0^\circ$, particularly for the largest AR (Fig. 4, B to D). This behavior is qualitatively consistent with our previous observations on U_{dd} because the phase transition is governed by the competition between the total onsite interaction—i. e. the sum of the dipolar and contact part—and the tunneling, U/J . For large ARs and $\theta = 90^\circ$, the DDI is mainly repulsive and thus strengthens the pinning of particles in the MI phase with respect to the SF phase, whereas the contrary happens for $\theta = 0^\circ$, where the SF phase is favored (Fig. 4B).

We systematically study the critical value of the lattice depth, $s_c(\theta)$, corresponding to the onset of the phase transition, for two dipole orientations, $\theta = 0^\circ$ and 90° , and various AR values. To determine $s_c(\theta)$, we use two independent methods. The first one, based on a double-line fit, identifies $s_c(\theta)$ as the point where the FWHM starts to grow, whereas the second method probes the visibility of the interference pattern (27, 41). The visibility is more reliable for large ARs, where the system undergoes a 3D phase transition and the side interference peaks have a larger contrast (27, 42). Figure 4E shows $\Delta s_c = s_c(0^\circ) - s_c(90^\circ)$ as a function of the AR. We find a substantial increase of Δs_c from negative to positive values, providing the opportunity to modify the phase diagram by tuning the strength and the sign of the DDI. In addition, we observe that Δs_c crosses zero at AR ≈ 1.2 (Fig. 4E). This value is unexpected because, if the DDI affected only the onsite interaction, one should record the same symmetric condition as measured in the frozen gas regime—i.e. AR = 1.07 (dotted line in Fig. 4E). However, at AR = 1.07, we still observe an angle dependence of the phase transition, with the MI phase favored for $\theta = 0^\circ$ (Fig. 4D). This behavior could be explained if the DDI also affected the tunneling

dynamics. In the eBH model, such a term appears in the form of a dipolar-interaction-driven tunneling mechanism—i.e., DIT—on top of the standard single-particle tunneling; see Eq. 1 and (29). For our typical experimental parameters, the DIT is predicted to be on the order of a few percent of the single-particle tunneling and exhibits an angle dependence (Fig. 1F and inset in Fig. 4E), which leads, for instance, to a reduction of the overall tunneling for out-of-plane orientation ($\theta = 0^\circ$) and thus to a shift of $s_c(0^\circ)$ to a smaller value. The presence of the DIT is consistent with our observations, as shown by the better agreement of our data with the mean-field calculations, including the DIT (solid line in Fig. 4E) with respect to the calculations without DIT (dotted line in Fig. 4E). For completeness, we checked with Monte Carlo calculations that the observed behavior cannot be explained by the NNI, as discussed in (27).

Quantum degenerate gases of magnetic lanthanide atoms in optical lattices offer an avenue to access the physics of strongly correlated systems for both bosonic and fermionic Hubbard dynamics in the presence of dipolar interactions, while building on the well-developed toolbox to prepare ultracold dense samples and to manipulate and measure these atomic gases. We have realized the extended Bose-Hubbard Hamiltonian with anisotropic onsite and offsite interactions, which reveal themselves in the excitation spectrum and in the many-body dynamics of the system. Our results show how to control the Hamiltonian terms with the dipole orientation and accomplish the long-awaited observation of NNI in Hubbard dynamics. An outstanding challenge for future experiments is the realization of many-body states in the lattice with spontaneously broken spatial symmetry due to NNI, such as the checkerboard and stripe phase (17). For our experimental parameters, the latter phase is expected to appear at temperatures in the few-nK regime (27). Dipolar interactions can be further increased by working with Feshbach molecules of magnetic lanthanides, essentially doubling the magnetic dipole moment (43), and subwavelength lattices (44, 45). Lanthanides offer opportunities to access the multitude of many-body phases predicted for dipolar quantum matter and are complemented by experimental developments with heteronuclear molecules and Rydberg atoms (2).

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SUPPLEMENTARY MATERIALS

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Supplementary Text
Figs. S1 to S5
Table S1
References (46–56)

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ELECTRONICS

Exploiting the colloidal nanocrystal library to construct electronic devices

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Synthetic methods produce libraries of colloidal nanocrystals with tunable physical properties by tailoring the nanocrystal size, shape, and composition. Here, we exploit colloidal nanocrystal diversity and design the materials, interfaces, and processes to construct all-nanocrystal electronic devices using solution-based processes. Metallic silver and semiconducting cadmium selenide nanocrystals are deposited to form high-conductivity and high-mobility thin-film electrodes and channel layers of field-effect transistors. Insulating aluminum oxide nanocrystals are assembled layer by layer with polyelectrolytes to form high-dielectric constant gate insulator layers for low-voltage device operation. Metallic indium nanocrystals are codispersed with silver nanocrystals to integrate an indium supply in the deposited electrodes that serves to passivate and dope the cadmium selenide nanocrystal channel layer. We fabricate all-nanocrystal field-effect transistors on flexible plastics with electron mobilities of 21.7 square centimeters per volt-second.

Electronics are increasingly pervasive as new applications in mobile, wearable, and implantable devices for communication, computation, and sensing are introduced. Many of these applications require high-mobility, low-cost, large-area, and flexible semiconductor devices and are therefore driving the development of new, solution-processable materials and fabrication processes to meet the demand. Colloidal, inorganic nanocrystals (NCs) are a promising class of solution-processable materials. Wet-chemical synthetic methods have enabled the preparation of colloidal, NC inks that are metallic (1, 2), semiconducting (3–5), and insulating (6). These NCs are attractive building blocks for the solution-based assembly of NC solids with emergent physical properties that are derived from the size, shape, and composition-dependent characteristics of the constituent NCs and the collective interactions between the NCs in the solid state (7, 8). The strength of the NC-NC interactions in solids can be tailored by the length and composition of NC surface ligands (9, 10). The recent introduction of compact, inorganic ligands and processes for NC surface exchange has led to demonstrations of strongly coupled, high-electrical conductivity and -mobility, metallic and semiconducting NC solids (11–13). These NC solids have been used to form the metallic wiring and semiconducting active layer of electronic (14–17) and optoelectronic (18–21) devices.

However, NCs are typically only used to form a single element in these devices, and the remainder of the device architecture is realized with

characteristically costly and slow, conventional vacuum-based deposition methods. Unlike conventional microelectronics, protocols for the integration of multiple, dissimilar, solution-processable NC materials to construct high-performance devices do not exist. Integration is a challenging feat and requires the development of orthogonal solution-based processes that do not detrimentally alter NC surface chemistry and that allow the complex stacking and patterning of NC thin films, and at the same time the design of chemically compatible, structurally stable, and physically cooperative materials and interfaces to achieve excellent device function.

Here we integrate metallic, semiconducting, and insulating NCs and design the materials, interfaces, and processes to enable the construction of all the components of high-performance, solution-processable, and flexible field-effect transistors (FETs) using colloidal NCs (Fig. 1A). Organic phase dispersions of Ag NCs, aqueous dispersions of Al₂O₃ NCs, and organic phase dispersions of CdSe NCs are selected for their properties and orthogonal processing to form and stack the gate electrode, gate insulator, and semiconducting channel layers of the FET, respectively. We introduce In in the form of colloidal NCs and codisperse the In NCs with Ag NCs to create a solution-processable NC ink used to construct the source and drain electrodes and to supply atomic In upon annealing that passivates and dopes the NC semiconducting channel. Surface exchange of the Ag and CdSe NCs with the compact ligand thiocyanate (SCN) is used here not only to create high-conductivity electrodes and high-mobility semiconductor channel layers, as we have shown previously (13, 22), but to allow the stacking of NC device layers. We modify the Ag, Al₂O₃, and CdSe NC thin-film surfaces with polyelectrolytes to control surface charge and passivation and to exploit their high dielectric constants, to engineer chemically compatible, structurally stable, and physically cooperative device layers. By designing the constituent materials, interfaces, and processes, we successfully integrate different NC building blocks using solution-based processes

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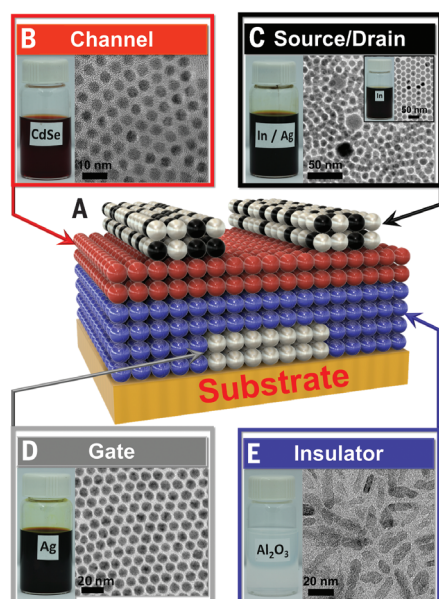


Fig. 1. Colloidal NC FET building blocks. (A) Schematic of an FET assembled from metallic, semiconducting, and insulating NCs. Photographs and TEM images of colloidal dispersions of (B) CdSe NCs forming the semiconducting channel, (C) In and Ag NC mixtures forming the source and drain electrodes, (D) Ag NCs forming the gate electrode, and (E) Al_2O_3 NCs forming the gate insulator layer.

to realize high-performance and flexible electronic devices, rivaling the high-mobility, low-hysteresis, and low-voltage operation of previously reported FETs fabricated from only a single-semiconductor NC layer but otherwise constructed using conventional processes.

Figure 1, B to E, shows photographs of the dispersions and transmission electron microscopy (TEM) images of the NC building blocks used as colloidal inks for FET fabrication. The process flow that enables us to integrate these NCs and to fabricate all-NC FETs is illustrated in Fig. 2A. To define Ag NC bottom gate electrodes, we lithographically pattern photoresist on a flexible Kapton substrate and then twice spin-coat a Ag NC dispersion (40 mg/ml) and immerse the Ag NC-coated substrate in a 1% NH_4SCN methanolic solution to exchange the native capping ligand (22). The resist is lifted off, yielding patterned, Ag NC gate electrodes 80 ± 10 nm in thickness.

We treat the Ag NC gate electrode surface with the polyelectrolytes poly(dimethyldiallyl ammonium chloride) (PDDA) and poly(styrenesulfonate) (PSS) and then deposit the Al_2O_3 NCs to form the gate insulator layer. Modification of the Ag NC gate electrode surface with the negatively charged PSS electrostatically enhances the adsorption of the positively charged Al_2O_3 NCs (fig. S1) and is necessary to assemble uniform Al_2O_3 NC layers (fig. S2). Subsequently, PSS and Al_2O_3 NCs are deposited layer by layer (LBL) by spin coating to construct the gate insulator layer (fig. S3). Here, modification of the positively charged Al_2O_3 NC surface with negatively charged PSS is required to grow the gate insulator layer, as the Al_2O_3 NCs cannot be sequentially deposited on themselves (fig. S4).

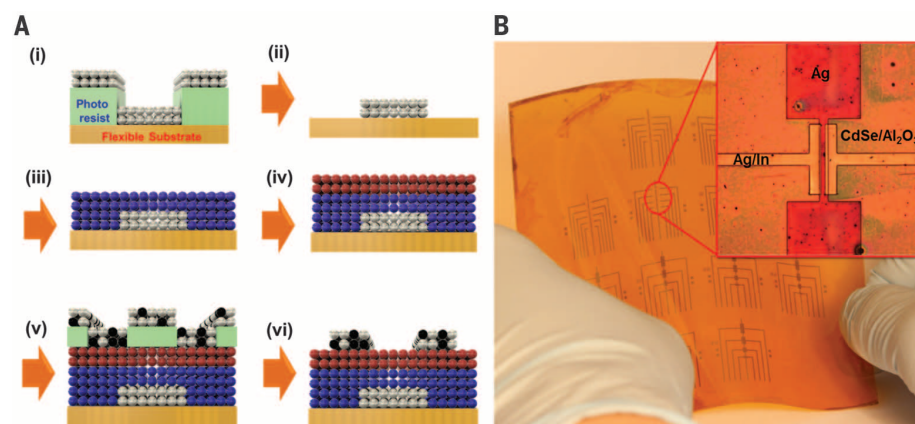


Fig. 2. Fabrication of flexible, all-NC FETs. (A) Schematic of the all-NC FET fabrication process on a flexible Kapton substrate via (i) photolithographic patterning and Ag NC spin-coating and ligand exchange; (ii) resist lift-off to define Ag NC gate electrodes; (iii) layer-by-layer spin coating of PDDA and PSS and then alternately Al_2O_3 NCs and PSS to grow the gate insulator layer; (iv) spin coating of SCN-exchanged CdSe NCs to form the semiconductor channel layer; (v) photolithographic patterning and Ag NC/In NC spin coating and ligand exchange; and (vi) resist lift-off to yield patterned electrodes. Devices are thermally annealed at 250°C for 10 min in the nitrogen glovebox. (B) Photograph of a flexible, all-NC FET array and (inset) a higher-magnification optical image of a single-NC FET.

To form low-leakage FETs, a gate insulator layer of ~ 80 nm is prepared by depositing three layers of a Al_2O_3 NC dispersion (25 mg/ml) alternately with PSS. The gate insulator layer is cured at 100°C for 30 min in air to remove residual solvent.

The organic ligands used to synthesize CdSe NCs are exchanged with compact SCN^- ligands (10, 13). SCN-exchanged CdSe NC dispersions (25 mg/ml) are spin-cast onto the Al_2O_3 NC gate insulator layer to form 60 ± 5 nm thick films (13). Before depositing the source and drain electrodes, the CdSe NC layer must be passivated to prevent delamination that is frequently observed during subsequent photolithographic patterning (15). Previously, we deposited an ultrathin (<1 nm) Al_2O_3 layer by vacuum-based, atomic-layer deposition (ALD) to provide the required passivation (15). Here we show that the solution-based deposition of a PDDA/PSS bilayer successfully prevents delamination (fig. S5). Structural, optical, electrochemical, and electrical characterization of the CdSe NC layer after the PDDA/PSS surface treatment shows that the treatment has little effect on the CdSe NC film, and analytical measurements show that surface SCN^- ligands are displaced by Cl^- introduced in the PDDA (23) (figs. S6 to S8). Surface halogenation in IV-VI NCs is known to passivate NC films electronically and to stabilize NC films toward processing in air (24). We show that surface halogenation through the PDDA/PSS treatment stabilizes the CdSe NC films in the “harsh” chemical environments needed for their solution-based device integration.

Finally, 100 ± 10 -nm-thick source and drain electrodes are patterned atop the CdSe NC layer, and the surface ligands are exchanged by immersion in a solution of NH_4SCN , following the same procedure used to define the gate electrode layer. Here we highlight that a codispersion of Ag NCs and In NCs is used to provide an In supply key to doping and passivating the CdSe NC channel. The fabricated devices are then thermally annealed at 250°C

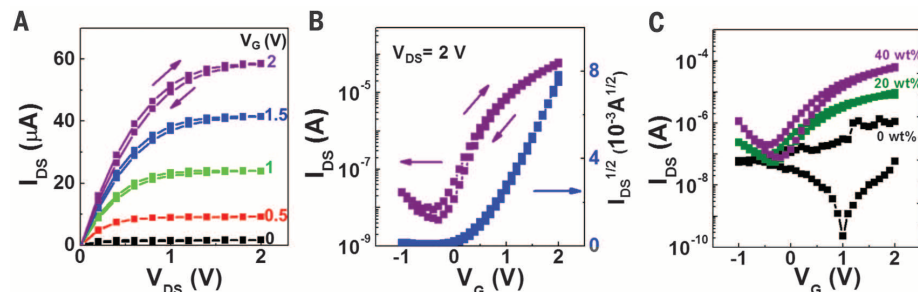
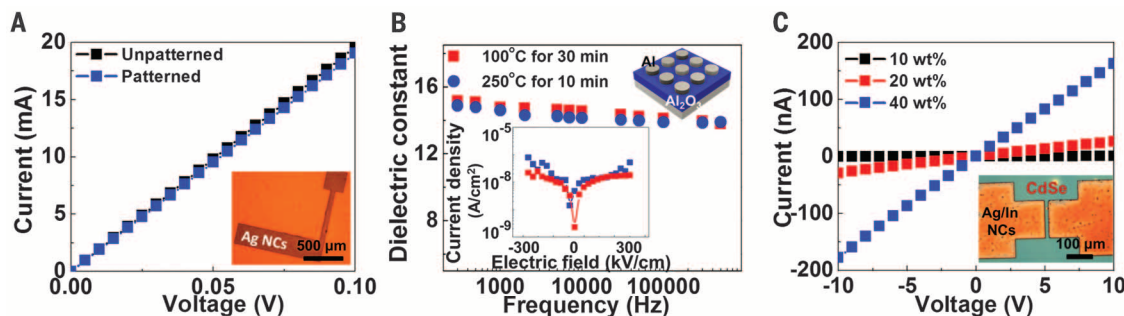
for 10 min in a nitrogen glovebox to densify the CdSe NC film and activate In diffusion from the colloidal NCs introduced in the source and drain electrodes. Figure 2B shows a photograph of an array of all-NC FETs fabricated on Kapton via this solution-based process; the inset shows a higher-magnification image detailing the NC-based metallic electrodes, semiconducting channel, and gate insulator layer that are stacked to form the FET.

To confirm the function of each NC component contributing to the integrated device performance, we examine their structural and electrical properties. The SCN-exchanged, Ag NC films are robust to immersion in the solvents used in photolithography, allowing the patterning of well-defined Ag NC electrodes (Fig. 3A, inset). The patterned ~ 80 -nm Ag NC films are highly uniform and crack-free, with a root mean square (RMS) roughness of ~ 7 nm (fig. S9) and a conductivity σ of 2.45×10^3 S/cm, similar to that of unpatterned Ag NC films (2.84×10^3 S/cm) (Fig. 3A). Annealing the Ag NC electrodes to 250°C , corresponding to the temperature seen in complete device fabrication, increases the conductivity to 2.38×10^4 S/cm, akin to that of vapor-deposited silver films (25).

The Al_2O_3 NC insulator layers prepared by LBL assembly are continuous and smooth (fig. S10A). The film thickness increases linearly and the RMS roughness increases marginally as the number of spin-coating cycles is increased from 1 to 10 (fig. S10B). We probe the frequency dependence of the dielectric constant of the Al_2O_3 NC insulator layers in a metal-insulator-metal configuration by capacitance-voltage measurements (Fig. 3B). The dielectric constant is ~ 15.1 at 50 Hz and shows $<5\%$ dispersion between 50 and 10^6 Hz for the 100°C , 30-min annealed Al_2O_3 NC layer, which is larger than the ~ 8 dielectric constant typical of Al_2O_3 layers grown by ALD (26), sputtering (27), and anodization (28). The increased dielectric constant is consistent with the incorporation of PSS, which has a high dielectric constant of ~ 30 to 120 (29). Upon further annealing

Fig. 3. NC device component characterization.

(A) Two-point resistivity measurements of patterned and unpatterned Ag NC films and (inset) photograph of a patterned Ag NC-based gate electrode. (B) Frequency-dependent dielectric constant for the Al_2O_3 NC dielectric layer; (inset, top) schematic of the metal-insulator-metal measurement configuration, and (inset, bottom) the leakage current density as a function of electric field. (C) Current-voltage characteristics and (inset) photograph of CdSe NC films with patterned Ag/In NC electrodes as a function of the In NC concentration.

**Fig. 4. Device characteristics of flexible, all-NC FETs.** (A) Output and (B) transfer characteristics of an all-NC FET (channel length $L = 30\ \mu\text{m}$, width $W = 450\ \mu\text{m}$). (C) Transfer characteristics of all-NC FETs fabricated with 0, 20, and 40 wt % of In NCs in the source and drain electrodes.

of the films at 250°C for 10 min to mimic the thermal processing seen in device fabrication, the dielectric constant is marginally smaller (~ 14.9), implying that the intervening PSS still contributes to the properties of the gate insulator layer in the completed FET. The increased dielectric constant of the Al_2O_3 NC insulator layer by the PSS allows more efficient charge accumulation in the semiconducting channel for low-voltage FET operation. The leakage current densities (Fig. 3B, inset) are $\sim 7.16 \times 10^{-7}\ \text{A}/\text{cm}^2$ and $\sim 2.2 \times 10^{-7}\ \text{A}/\text{cm}^2$ at an applied electric field of $300\ \text{kV}/\text{cm}$ for 80-nm films annealed at 100°C for 30 min and further annealed at 250°C for 10 min, respectively. The thin, yet low-leakage and high-dielectric constant Al_2O_3 NC-based gate insulator layers enable our realization of low-voltage and high-performance, flexible NC electronics.

To form high-mobility CdSe NC FETs, we previously introduced thermal evaporation and diffusion of elemental In to form ohmic contacts by n-doping the contact regions and to electronically passivate and dope the NC channel (15). Here, we introduce colloidal In NCs into the Ag NC dispersion used to form the source and drain electrodes as a solution-based, NC route to provide the supply of atomic In upon thermal annealing. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is used to monitor the lateral and depth profile of In in and across the FET channel. Before annealing, no In is observed in the channel; however, after annealing, In is found uniformly distributed in the FET channel (fig. S11). The electrical characteristics of CdSe NC layers patterned with In NC-containing electrodes and annealed at 250°C for 10 min (Fig. 3C) show ohmic behavior at low voltages with conductivities that increase from $\sim 10^{-7}\ \text{S}/\text{cm}$ for 0

weight % (wt %) In NCs (fig. S12) to $\sim 2.7 \times 10^{-4}\ \text{S}/\text{cm}$ at 40 wt % of In NCs. By controlling the concentration of colloidal In NCs mixed with Ag NCs to form the source and drain electrodes, we can design the doping level of the CdSe NC channel.

We integrate the metallic, semiconducting, and insulating NCs to construct all-inorganic NC FETs, as depicted in the schematic in Fig. 1 and shown by scanning electron micrographs in fig. S13. Figure 4, A and B, shows representative output and transfer characteristics of our all-NC, solution-processed, flexible FETs fabricated from 40 wt % In NCs mixed in the Ag NC source and drain electrodes, a SCN-exchanged CdSe NC semiconducting channel, an Al_2O_3 NC gate insulator layer, and a Ag NC gate electrode. The devices operate at low voltages and exhibit well-behaved n-type FET characteristics in the linear and saturation regimes (Fig. 4A) with an average electron mobility of $\mu = 21.7 \pm 4.5\ \text{cm}^2/\text{V}\cdot\text{s}$, a threshold voltage $V_T = 0.36 \pm 0.21\ \text{V}$, and a subthreshold swing $S = 0.31 \pm 0.05\ \text{V}/\text{decade}$, characterized from four independently prepared devices (fig. S14). The FETs show low hysteresis, as characterized by $\Delta V_T = 0.21 \pm 0.06\ \text{V}$ at a drain-source voltage $V_{DS} = 2\ \text{V}$. The low-voltage and low-hysteresis FET operation is attributed to the high capacitance ($0.176\ \mu\text{F}/\text{cm}^2$) of the Al_2O_3 NC insulator layer and the low-interface trap density at the Al_2O_3 NC/CdSe NC interface. The transfer characteristics of all-NC FETs fabricated with lower concentrations of 0 and 20 wt % In NCs in the Ag NC source and drain electrodes show lower on-currents and current modulations (Fig. 4C), consistent with their low doping levels (fig. S15). Increasing the In NC concentration to 40 wt % in the Ag NC-based source and drain electrodes increases the on-current

and current modulation to realize all-solution-processed CdSe NC FETs with a high performance similar to that of other state-of-the-art NC FETs fabricated by vacuum-based deposition of the electrodes and gate insulator layer (11, 13, 15, 16).

In conclusion, we report all-NC FETs through the solution-based deposition, patterning, and integration of metallic, semiconducting, and insulating colloidal NC inks to construct the device components. The high performance of these all-NC FETs demonstrates the competitive advantage of colloidal NC materials for large-area, low-cost, and flexible electronics, eliminating the need for conventional vacuum-processed materials and setting the stage to exploit colloidal NC materials for additive manufacturing of devices. The materials integration and fabrication processes reported here may be applied more broadly to the construction of NC-based electronic, optoelectronic, and thermoelectric devices from the wide range of size, shape, and compositionally designed colloidal NC building blocks.

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SUPPLEMENTARY MATERIALS

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ASTROCHEMISTRY

Ribose and related sugars from ultraviolet irradiation of interstellar ice analogs

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Ribose is the central molecular subunit in RNA, but the prebiotic origin of ribose remains unknown. We observed the formation of substantial quantities of ribose and a diversity of structurally related sugar molecules such as arabinose, xylose, and lyxose in the room-temperature organic residues of photo-processed interstellar ice analogs initially composed of H₂O, CH₃OH, and NH₃. Our results suggest that the generation of numerous sugar molecules, including the aldopentose ribose, may be possible from photochemical and thermal treatment of cosmic ices in the late stages of the solar nebula. Our detection of ribose provides plausible insights into the chemical processes that could lead to formation of biologically relevant molecules in suitable planetary environments.

DNA is the genetic source code for all known living organisms. It is currently thought that DNA evolved from a primordial ribonucleic acid RNA world state (1, 2), in which ribose chemically binds and orien-

tates the complementary purine and pyrimidine nucleobases for efficient base pairing. Ribose thereby forms the essential part of the RNA backbone. However, ribose is difficult to form, and the source of the ribose subunits in the sugars that consti-

tute the key stereodictating elements in nucleic acid structure remained unknown (3, 4). We describe here the identification of precursor molecules, including ribose, in simulated precometary ices using the sensitive two-dimensional gas chromatography time-of-flight mass spectrometry (GC×GC-TOFMS) technique.

Our astrophysical scenario involves the simulation of the photo- and thermo-chemistry of precometary ices. It is based on the assumption that planetesimals (including asteroids, comets, and the parent bodies of meteorites) were formed in the solar nebula from the aggregation of icy grains

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Fig. 1. Aldoses and ketoses as identified in a sample generated under simulated precometary conditions. The structures of sugar alcohols, mono-saccharides, and saccharinic acids are indicated along with the amount of the identified analyte in the simulated ice sample. Identified C-6 analytes are not included. The Fischer projections indicate the D-enantiomer form only. ppm, parts per million by mass; q.l., quantification limit; d.l., detection limit.

	Aldoses				Ketoses	
C-2	$\begin{array}{c} \text{R} \\ \\ \text{CH}_2\text{OH} \end{array}$ R = CH ₂ OH, Ethylene glycol (550 ppm) = CHO, Glycolaldehyde (2390 ppm) = COOH, Glycolic acid (6330 ppm)					
C-3	$\begin{array}{c} \text{R} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OH} \end{array}$ R = CH ₂ OH, Glycerol (2860 ppm) = CHO, Glyceraldehyde (302 ppm) = COOH, Glyceric acid (2440 ppm)				$\begin{array}{c} \text{CH}_2\text{OH} \\ \\ \text{C}=\text{O} \\ \\ \text{CH}_2\text{OH} \end{array}$ Dihydroxyacetone (540 ppm)	
C-4	$\begin{array}{c} \text{R} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OH} \end{array}$ R = CH ₂ OH, Erythritol (5070 ppm) = CHO, Erythrose (< q.l.) = COOH, Erythronic acid (960 ppm)		$\begin{array}{c} \text{R} \\ \\ \text{HO}-\text{C}-\text{H} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OH} \end{array}$ R = CH ₂ OH, Threitol (7200 ppm) = CHO, Threose (< d.l.) = COOH, Threonic acid (840 ppm)		$\begin{array}{c} \text{CH}_2\text{OH} \\ \\ \text{C}=\text{O} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OH} \end{array}$ Erythrulose (37 ppm)	
C-5	$\begin{array}{c} \text{R} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OH} \end{array}$ R = CH ₂ OH, Ribitol (560 ppm) = CHO, Ribose (260 ppm) = COOH, Ribonic acid (82 ppm)	$\begin{array}{c} \text{R} \\ \\ \text{HO}-\text{C}-\text{H} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OH} \end{array}$ Arabinitol (1150 ppm) Arabinose (200 ppm) Arabinic acid (165 ppm)	$\begin{array}{c} \text{R} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{HO}-\text{C}-\text{H} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OH} \end{array}$ Xylitol (630 ppm) Xylose (240 ppm) Xylonic acid (67 ppm)	$\begin{array}{c} \text{R} \\ \\ \text{HO}-\text{C}-\text{H} \\ \\ \text{HO}-\text{C}-\text{H} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OH} \end{array}$ Arabitol (1150 ppm) Lyxose (145 ppm) Lyxonic acid (140 ppm)	$\begin{array}{c} \text{CH}_2\text{OH} \\ \\ \text{C}=\text{O} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OH} \end{array}$ Ribulose (2010 ppm)	$\begin{array}{c} \text{CH}_2\text{OH} \\ \\ \text{C}=\text{O} \\ \\ \text{HO}-\text{C}-\text{H} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OH} \end{array}$ Xylulose (470 ppm)

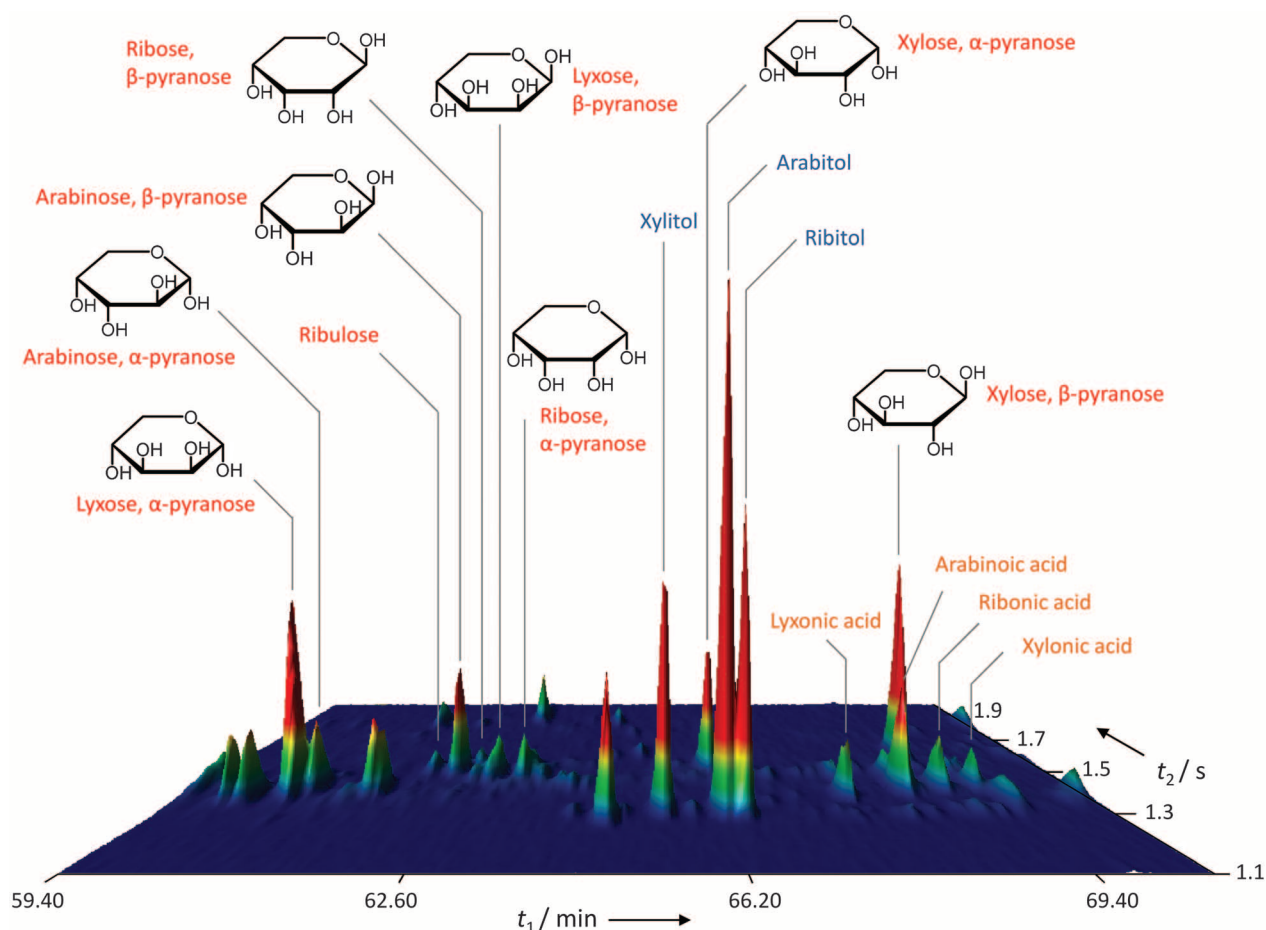
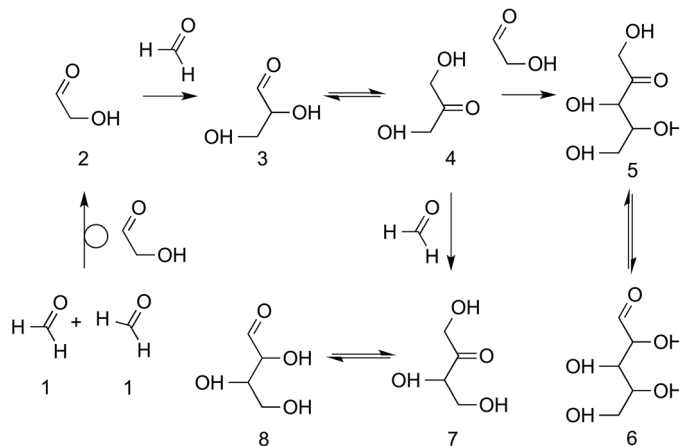


Fig. 2. Multidimensional gas chromatogram showing ribose and other monosaccharides in the organic residue from an evolved precometary ice analog. See also fig. S1 and movie S1 (7). The atomic mass units 206 and 294 were selected for the multidimensional chromatographic representation. D- and L-enantiomers of the monosaccharides were not resolved.

Fig. 3. The formose reaction.

Formaldehyde **1** condenses under autocatalytic reaction kinetics to form glycolaldehyde **2**, which undergoes an aldol reaction by forming glyceraldehyde **3**. Dihydroxyacetone **4** is formed by aldose-ketose isomerization of **3** and reacts with **2**, forming pentulose **5**, which isomerizes to an aldopentose **6** such as ribose. In an alternative pathway, dihydroxyacetone **4** reacts with **1**, producing ketotetrose **7** and aldotetrose **8** (18).



complex chemistry in the condensed phase. We empirically simulated this process in the laboratory at low temperature ($T = 78$ K) and low pressure ($p = 10^{-7}$ mbar). Volatile molecules H_2O , $^{13}\text{CH}_3\text{OH}$, and NH_3 in a 10:3.5:1 ratio were condensed on the cooled surface, while being irradiated with ultraviolet photons, and then warmed up to room temperature, leading to the formation of an organic residue (7). ^{13}C isotopic labeling of the methanol allowed us to distinguish between the reaction products and potential contamination during manipulation of the sample and through the analytical procedure. A precise description of the experimental setup is provided in the supplementary materials.

The samples were extracted with water, derivatized, and submitted to GC×GC-TOFMS analysis (8). The analytical multidimensional separation apparatus consisted of a 0.25-mm capillary column with a β -cyclodextrin stationary phase in the first dimension and a polyethylene glycol capillary column of 100 μm inner diameter in the second dimension. A cryogenic modulator connected both capillary columns (7). A reflectron time-of-flight (TOF) mass spectrometer has been used for recording retention times and mass

already present and processed at the late molecular cloud stages. This also took place within the protoplanetary disk phase, owing to vertical/radial mixing of the protostellar materials (5). As a model, these dust particles are composed of silicate (or

carbon) grains surrounded by icy mantles containing H_2O , CH_3OH , NH_3 , and other volatile species (6). In this environment, dust particles and icy mantles are expected to be subjected to ultraviolet photons and cosmic-ray irradiation, leading to a

Table 1. Identified sugars and related organic compounds in interstellar ice analogs initially composed of H₂O:¹³CH₃OH:NH₃, #C, quantity of carbon atoms; R_{t1}, GC×GC retention time 1st dimension; R_{t2}, GC×GC retention time 2nd dimension.

#C	Compound	R _{t1} (min)	R _{t2} (sec)	MS-fragmentation/ ¹³ C sample		MS-fragmentation/ ¹² C standard	
				(M ⁺⁺)	Other important ions (m/z)	(M ⁺⁺)	Other important ions (m/z)
Monosaccharides							
3	L-Glyceraldehyde	33.48	1.78	237	207, 206, 191, 147, 119, 104, 73, 29	234	205, 204, 189, 147, 117, 103, 73, 28
	D-Glyceraldehyde	34.03	1.74		see L-Glyceraldehyde		
	Dihydroxyacetone	43.37	1.88	237	222, 191, 147, 104, 73	234	219, 189, 147, 103, 73
4	D,L-Erythrose, α-furanose	49.46	1.52	340	235, 221, 220, 206, 192, 147, 104, 73	336	231, 218, 217, 203, 191, 147, 103, 73
	D,L-Erythrulose	54.51	1.82	340	309, 235, 207, 191, 147, 119, 104, 73	336	306, 231, 205, 189, 147, 117, 103, 73
5	D,L-Lyxose, α-pyranose	60.90	1.41	443	see Ribose		
	D,L-Arabinose, α-pyranose	61.15	1.42		see Ribose		
	D,L-Ribulose, ketol	62.50	1.46	443	339, 310, 249, 220, 192, 147, 132, 104, 73	438	335, 307, 245, 218, 191, 147, 129, 103, 73
	D,L-Arabinose, β-pyranose	62.85	1.51		see Ribose		
	D,L-Ribose, β-pyranose	63.15	1.45	443	308, 294, 220, 206, 192, 147, 119, 104, 103, 73	438	305, 291, 217, 204, 191, 147, 117, 103, 101, 73
	D,L-Lyxose, β-pyranose	63.40	1.48	443	see Ribose		
	D,L-Ribose, α-pyranose	63.70	1.46	443	308, 294, 220, 206, 192, 147, 119, 103, 73	438	305, 291, 217, 204, 191, 147, 117, 103, 101, 73
	D,L-Xylulose, ketol*†	64.30	1.54	443	339, 310, 249, 220, 192, 147, 132, 104, 73	438	335, 307, 245, 218, 191, 147, 129, 103, 73
	D,L-Xylose, α-pyranose	66.20	1.52	443	see Ribose		
	D,L-Xylose, β-pyranose	69.04	1.61	443	see Ribose		
Sugar acids (saccharinic acids)							
3	D,L-Glyceric acid	44.42	1.64	325	310, 294,‡ 220, 207, 191, 177, 147, 119, 103, 73	322	307, 292,‡ 217, 205, 189, 175, 147, 117, 102, 73
4	D,L-Erythronic acid	56.96	1.47	428	413, 382, 294,‡ 222, 207, 147, 104, 73	424	409, 379, 292,‡ 220, 205, 147, 103, 73
	D,L-Threonic acid	58.10	1.49	428	413, 382, 294,‡ 222, 207, 147, 104, 73	424	409, 379, 292,‡ 220, 205, 147, 103, 73
5	D,L-Lyxonic acid†	67.79	1.39	531	see Ribonic acid		
	D,L-Arabinic acid†	68.59	1.41	531	see Ribonic acid		
	D,L-Ribonic acid†	69.09	1.41	531	337, 310, 294,‡ 279, 220, 207, 191, 147, 119, 104, 73	526	333, 307, 292,‡ 277, 217, 205, 189, 147, 117, 103, 73
	D,L-Xylonic acid†	69.54	1.42	531	see Ribonic acid		
Sugar alcohols							
3	Glycerol	40.58	1.43	311	296, 221, 207, 147, 119, 104, 73	308	293, 218, 205, 147, 117, 103, 73
4	D,L-Threitol	54.56	1.38	414	324, 310, 295, 220, 207, 191, 147, 119, 104, 73	410	320, 307, 293, 217, 205, 189, 147, 117, 103, 73
	Erythritol	54.86	1.38	414	324, 310, 295, 220, 207, 191, 147, 119, 104, 73	410	320, 307, 293, 217, 205, 189, 147, 117, 103, 73

continued on next page

#C	Compound	R_{t1} (min)	R_{t2} (sec)	MS-fragmentation/ ^{13}C sample		MS-fragmentation/ ^{12}C standard	
				(M^{++})	Other important ions (m/z)	(M^{++})	Other important ions (m/z)
5	D,L-Xylitol	65.54	1.33	517	337, 323, 310, 220, 207, 191, 147, 119, 104, 73	512	332, 319, 307, 217, 205, 189, 147, 117, 103, 73
	D,L-Arabitol	66.29	1.32	517	337, 323, 310, 220, 207, 191, 147, 119, 104, 73	512	332, 319, 307, 217, 205, 189, 147, 117, 103, 73
	D,L-Ribitol (Adonitol)*†	66.49	1.31	517	337, 323, 310, 220, 207, 191, 147, 119, 104, 73	512	332, 319, 307, 217, 205, 189, 147, 117, 103, 73
6	D,L-Mannitol	82.54§	1.63	620	426, 323, 310, 220, 207, 147, 104, 73	614	421, 319, 307, 217, 205, 147, 103, 73
	D,L-Sorbitol	83.24§	1.65	620	426, 323, 310, 220, 207, 147, 104, 73	614	421, 319, 307, 217, 205, 147, 103, 73
	D,L-Allitol*	83.49§	1.67	620	see Mannitol		
	D,L-Dulcitol (Galactitol)	83.69§	1.67	620	426, 323, 310, 220, 207, 147, 104, 73	614	421, 319, 307, 217, 205, 147, 103, 73
	D,L-Talitol (Altritol)*	84.04§	1.67	620	see Mannitol		
Branched-chain sugar acids and polyols							
4	2-Methylglycerol†	40.03	1.31	326	311, 235, 222, 147, 104, 73	322	307, 232, 219, 147, 103, 73
	2-Methylglyceric acid*	43.02	1.48	340	325, 309, ‡ 296, 236, 222, 206, 191, 147, 104, 73	336	321, 306, ‡ 293, 233, 219, 203, 189, 147, 103, 73
	2-Hydroxymethylglycerol*	52.81	1.33	414	310, 220, 191, 147, 132, 104, 73		
5	2-Hydroxymethyltetritol*	64.90	1.29	517	323, 310, 220, 147, 104, 73		
Hydroxycarboxylic acids							
2	Glycolic acid	31.39	1.69	222	207, 178, ‡ 162, 147, 104, 73	220	205, 177, ‡ 161, 147, 103, 73
3	D-Lactic acid	24.94	1.66	237	222, 193, 147, 119, 103, 73	234	219, 191, 147, 117, 101, 73
	L-Lactic acid	25.24	1.66	237	222, 193, 147, 119, 103, 73	234	222, 191, 147, 117, 101, 73
	3-Hydroxypropanoic acid	36.63	1.60	237	222, 178, 147, 118, 103, 73	234	219, 177, 147, 116, 101, 73
4	2-Hydroxyisobutyric acid	24.00	1.52	252	237, 208, ‡ 147, 134, 73	248	233, 205, ‡ 147, 131, 73
	(R)-2-Hydroxybutyric acid	29.54	1.65	252	237, 208, ‡ 147, 134, 73	248	233, 205, ‡ 147, 131, 73
	(S)-2-Hydroxybutyric acid	29.79	1.63	252	237, 208, ‡ 147, 134, 73	248	233, 205, ‡ 147, 131, 73
	3-Hydroxyisobutyric acid†	34.73	1.58	252	237, 221, 208, 178, 162, 147, 104, 73	248	233, 218, 205, 177, 158, 147, 103, 73
	(R)-3-Hydroxybutyric acid	35.63	1.57	252	237, 206, ‡ 193, 147, 119, 73	248	233, 204, ‡ 191, 147, 117, 73
	(S)-3-Hydroxybutyric acid	36.13	1.55				
	4-Hydroxybutyric acid	42.52	1.62	252	237, 206, 147, 119, 73	248	233, 204, 147, 117, 73
	D,L-Malic acid	55.81	1.77	354	339, 310, 249, 236, 191, 147, 119, 73	350	335, 307, 245, 233, 189, 147, 117, 73
Dicarboxylic acids							
4	Succinic acid	48.37	2.05	266	251, 176, 147, 132, 73	262	247, 172, 147, 129, 73
	Fumaric acid	51.76	1.96	264	249, 220, 147, 118, 73	260	245, 217, 147, 115, 73
Miscellaneous							
2	Ethylene glycol	16.00	1.38	208	193, 147, 104, 73	206	191, 147, 103, 73
	Oxamic acid*†	37.68	1.61	235	220, 191, 147, 117, 73	233	218, 190, 147, 116, 73
	Glycolaldehyde¶	36.18	2.16		304, 288, 215, 191, 147, 73		302, 286, 214, 189, 147, 73
		36.48	2.12		304, 288, 215, 191, 147, 73		302, 286, 214, 189, 147, 73
3	D,L-1,3-Propanediol	27.69	1.36	223	208, 178, 147, 133, 73	220	205, 177, 147, 130, 73

*Identification is based on literature data on the formose reaction (19, 20), former ice simulation experiments, as well as chromatographic retention properties of sugar and sugar-related molecules (7). †Identification is based on National Institute of Standards and Technology mass spectral database and retention time prediction. ‡McLafferty and McLafferty-type (ester migration) rearrangement. §Retention time shift by +3 min because of changes in column flow. ||Temperature program of first dimension: 40°C (1 min), 10°C min⁻¹, 80°C (10 min), 2°C min⁻¹, 180°C (5 min). ¶Stereoisomers of dimeric or anhydrol form.

spectra. The GC×GC-TOFMS analysis in combination with our derivatization protocol (7) provided the simultaneous resolution and identification of the sugar and sugar-related compounds, which has not been possible with classical chromatographic devices because of coelution of analytes.

We detected aldoses and ketoses in these simulated interstellar ice analogs as schematically represented by the Fischer projections in Fig. 1. The monosaccharides ribose, arabinose, xylose, and lyxose belong to the aldopentoses; ribulose and xylulose are ketopentoses. The aldopentoses were resolved and identified in

both α - and β -pyranose form (Fig. 2), and the ketopentoses show ketol form. Besides aldopentoses and ketopentoses, we identified the ketotetrose erythrulose. Mass spectra of the analytes and the interpretation of characteristic fragmentation pathways leading to unambiguous identification are provided (supplementary text),

along with details on the quantification of the analytes.

The corresponding saccharinic acids (sugar acids) were identified. We also detected the corresponding sugar alcohols, along with threitol, erythritol, mannitol, and sorbitol. We further found the related series of hydroxycarboxylic acids.

A systematic representation of the identified molecular species is given in Table 1, which indicates the analytes' retention times in the first and second chromatographic dimension and mass spectra of analyzed sample and standard. The analytes detected in the organic residue were found to contain ^{13}C isotopes, whereas the standard samples used for identification were made of the natural isotopic composition dominated by ^{12}C (figs. S3 to S5). We therefore exclude experimental contamination and conclude that all described analytes were formed by the H_2O , $^{13}\text{CH}_3\text{OH}$, and NH_3 volatile reactants that were deposited under simulated interstellar precometary conditions (fig. S2).

The molecular complexity in the evolved ices reported here suggests a far larger similarity to meteoritic organic materials than previously assumed. Thus, molecular evolution of meteoritic organic species available for planetary environments could have benefited from accretion of protostellar material. The sugars, dicarboxylic acids, C-4 hydroxycarboxylic acids, and branched-chain molecules described here have not previously been identified, although aldehydes, including glycolaldehyde and glyceraldehyde, have been found in similar laboratory residues (9), along with lower hydroxycarboxylic acids (10). Sugar-related alcohols and acids have previously been detected in the Murchison meteorite (11). Glycolaldehyde has been found sublimating from the nucleus of comet Lovejoy (C/2014 Q2) (12) and by means of millimeter-wave rotational transitions in emission toward the Galactic center source Sagittarius B2(N) (13). Glycolaldehyde was recently also detected in solar-type protostars (14). Moreover, glycolaldehyde and ethylene glycol were characterized by means of infrared spectroscopy in radiation chemistry of laboratory ices (15).

The diversity of sugar molecules formed from H_2O , $^{13}\text{CH}_3\text{OH}$, and NH_3 under interstellar conditions can be understood in the framework of a photochemically initiated formose-type reaction. Reactant and intermediate species of the formose reaction (16–18) are formaldehyde and glycolaldehyde that undergo aldol condensations to produce hydroxyl aldehydes and hydroxyl ketones with linear and branched structures (Fig. 3 and fig. S6). As in our case, sugar alcohols and sugar acids typically accompany the sugars formed by the formose reaction. We further detected the branched chain molecules hydroxymethylglycerol and hydroxymethyltetritol, which indicate a formose-type reaction mechanism (19, 20). The classical formose reaction is known to require a divalent metal catalyst such as Ca^{2+} , Pb^{2+} , or Tl^{2+} for the stabilizing effect of chelating ions on enediols (18), a condition not needed in the present work. Final steps of the formose-type reaction might

have occurred above 78 K during temperature increase of the sample to room temperature, in which reactants diffuse and react within the labile and sublimating ice matrix.

The parts-per-million values of the photo-products described in Fig. 1 sum up to greater than 35,000 (table S1), which corresponds to a value $>3.5\%$ (by mass) (7). Thus, sugars, sugar alcohols, and sugar acids are present notably above trace or ultratrace quantities and are therefore considered major molecular constituents of the condensed interstellar organics. The relative yield of ribose, which is known to be very small in the classical formose reaction, resulted in higher values during our cold scenario. We regard this result as central for an implication for prebiotic chemistry because it shows that even at this initial level of molecular “simplicity,” we observe an autocatalytic reaction. This, in turn, may be interpreted as a link between astrochemistry and astrobiology. The acquisition of kinetic data and enantioseparation will be necessary to rationalize the reaction mechanism of the sugar formation and to search for asymmetric autocatalytic effects.

The identification of ribose in the organic residues obtained from evolved precometary ices does not necessarily indicate the prebiotic evolutionary pathway. Besides ribose, we identified threose in its oxidized and reduced form, threonic acid and threitol, respectively. Threose is the molecular component of threose nucleic acid (TNA), a nucleic acid analog discussed to have preceded RNA (21). We further detected glycol and glycerol, which provide the molecular backbones of glycol and glycerol nucleic acids (GNAs) (22, 23). The molecular building blocks of peptide nucleic acids (PNAs), *N*-(2-aminoethyl) glycine and 2,4-diaminobutanoic acid, were previously detected in organic residues from similar ice experiments (24). In this experimental frame, precursors of both proteins (amino acids) (25) and genetic material (sugars and their derivatives) are produced in large amounts so that if delivered from meteorites in the Earth's environment, their coevolution may be considered one of the standing issues in prebiotic chemistry (26).

Our samples are fully soluble in water (as well as in other polar solvents), a condition that is essential for any further prebiotic activity. In aqueous solution, sugar molecules, including ribose, develop chemical equilibria between the open form (Fig. 1), the cyclic furanose and pyranose forms (Fig. 2), and in between the various constitutional isomers such as ribose and arabinose. Our experiments demonstrate plausible physical and chemical environmental conditions that allow for abiotic ribose synthesis.

Our findings support the identification of organic molecules in cometary samples taken in situ by the Philae Lander (27, 28) part of the cometary Rosetta mission (29). Our analytical results demonstrate the usefulness of multidimensional chromatographic techniques for the chemical analyses of extraterrestrial samples, which are often available only in small quantities,

in which sugar molecules including ribose may be present.

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SUPPLEMENTARY MATERIALS

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QUANTUM CRITICALITY

Quantum criticality with two length scales

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The theory of deconfined quantum critical (DQC) points describes phase transitions at absolute temperature $T = 0$ outside the standard paradigm, predicting continuous transformations between certain ordered states where conventional theory would require discontinuities. Numerous computer simulations have offered no proof of such transitions, instead finding deviations from expected scaling relations that neither were predicted by the DQC theory nor conform to standard scenarios. Here we show that this enigma can be resolved by introducing a critical scaling form with two divergent length scales. Simulations of a quantum magnet with antiferromagnetic and dimerized ground states confirm the form, proving a continuous transition with deconfined excitations and also explaining anomalous scaling at $T > 0$. Our findings revise prevailing paradigms for quantum criticality, with potential implications for many strongly correlated materials.

In analogy with classical phase transitions driven by thermal fluctuations, condensed matter systems can undergo dramatic changes as parameters regulating quantum fluctuations are tuned at low temperatures. Some of these quantum phase transitions can be theoretically understood as straightforward generalizations of thermal phase transitions (1, 2), where, in the conventional Landau-Ginzburg-Wilson (LGW) paradigm, states of matter are characterized by order parameters. Many strongly correlated quantum materials, however, seem to defy such a description.

In two-dimensional (2D) quantum magnets (3, 4), a promising proposal that extends beyond LGW physics is the theory of deconfined quantum critical (DQC) points, in which the order parameters of the antiferromagnetic (Néel) state and the competing dimerized state (the valence-bond-solid or VBS state) are not fundamental variables but composites of fractional degrees of freedom that carry a spin $S = 1/2$. These spinons are condensed and confined, respectively, in the Néel and VBS states, and they become deconfined at the DQC point separating the two states. Establishing the applicability of the still-controversial DQC scenario would be of great interest in condensed matter physics, where it may play an important role in strongly correlated systems such as cuprate superconductors (5). There are also DQC analogs to quark confinement and other aspects of high-energy physics (e.g., an emergent gauge field and the Higgs mechanism and boson particle) (6).

The DQC theory represents the culmination of a large body of field-theoretic works on VBS states and quantum phase transitions out of the Néel

state (2, 7–10). The postulated $SU(N)$ (special unitary groups of N dimensions) $NCCP^{N-1}$ (non-compact complex projective) action can be solved when $N \rightarrow \infty$ (5, 11, 12), but nonperturbative numerical simulations are required to study small N . The most natural physical realizations of the Néel-VBS transition for electronic $SU(2)$ spins are frustrated quantum magnets (9), which are notoriously difficult to study numerically (13, 14). Other models were therefore pursued. In the J - Q model (15), the Heisenberg exchange J between $S = 1/2$ spins is supplemented by a VBS-inducing four-spin term Q , which is amenable to efficient quantum Monte Carlo (QMC) simulations (15–23). Although many results for the J - Q model support the DQC scenario, it has not been possible to draw definite conclusions because of violations of expected scaling relations that affect many properties. Similar anomalies were later observed in 3D loop (24) and dimer (25) models, which are also potential realizations of the DQC point. Simulations of the $NCCP^1$ action as well have been hard to reconcile with the theory (21, 26, 27).

One interpretation of the unusual scaling behaviors is that the transitions are first order, as is generally required within the LGW framework for order-order transitions where unrelated symmetries are broken. The DQC theory then would not apply to any of the systems studied so far, thus casting doubts on the entire concept (17, 21, 26). In other interpretations, the transition is continuous, but unknown mechanisms cause strong corrections to scaling (18, 27, 28) or modify the scaling more fundamentally in some as yet unexplained way (19, 24). The enigmatic current state of affairs is summarized well in (24).

Here we show that the DQC puzzle can be resolved based on a finite-size scaling ansatz that includes the two divergent length scales of the theory: the standard correlation length ξ , which captures the growth of both order parameters ($\xi_{\text{Néel}} \propto \xi_{\text{VBS}}$), and a faster-diverging length ξ' , which is associated with the thickness of VBS domain walls and spinon confinement (the size

of a spinon bound state). We show that, contrary to past assumptions, ξ' can govern the finite-size scaling even of magnetic properties that are sensitive only to ξ in the thermodynamic limit. Our simulations of the J - Q model at low temperatures and in the lowest $S = 1$ (two-spinon) excited state demonstrate complete agreement with the two-length scaling hypothesis.

Consider first a system with a single divergent correlation length $\xi \propto |\delta|^{-\nu}$, where $\delta = g - g_c$ is the distance to a phase transition that is driven by quantum fluctuations arising from noncommuting interactions controlled by g at absolute temperature $T = 0$, g_c is the critical value of the control parameter g , and ν is a critical exponent. In finite-size scaling theory (29), for a system of linear size L (volume L^d in d dimensions), close to $\delta = 0$, a singular quantity A takes the form

$$A(g, L) = L^{-\kappa/\nu} f(\delta L^{1/\nu}, L^{-\omega}) \quad (1)$$

where the exponents κ , ν , and ω are tied to the universality class, κ also depends on A , and the scaling function $f \rightarrow \text{constant}$ (up to corrections $\propto L^{-\omega}$) when $\delta \rightarrow 0$. We assume $T \propto L^{-z}$, z being the dynamic exponent (or, alternatively, $T = 0$), so that scaling arguments depending on T have been eliminated.

The form in Eq. 1 fails for some properties of the J - Q model (18, 19, 22) and other DQC candidate systems (24–26). A prominent example is the spin stiffness ρ_s , which, for an infinite 2D system in the Néel phase, should scale as $\rho_s \propto \delta^{2\nu}$ with $z = 1$ (1, 3, 4). To eliminate the size dependence when $\delta \neq 0$ and $L \rightarrow \infty$ in Eq. 1, it is necessary to have $\kappa = z\nu$ and $f(x, L^{-\omega}) \propto x^{z\nu}$ for large $x = \delta L^{1/\nu}$. Thus, $\rho_s(\delta = 0, L) \propto L^{-z}$ and, if $z = 1$, $L\rho_s \rightarrow \text{constant}$ when $L \rightarrow \infty$. However, $L\rho_s(L)$ at criticality instead appears to diverge slowly (17, 18, 21). At first glance, this might suggest $z < 1$, but other quantities (e.g., the magnetic susceptibility) instead behave as if $z > 1$ (30). Strong scaling corrections have been suggested as a way to resolve this paradox (18, 19, 28). Claims of a weak first-order transition have also persisted (21, 26, 27), although the continuous DQC scenario is supported by the absence of any of the usual first-order signals (e.g., the Binder cumulant does not exhibit any negative peak) (18, 24).

To explain the scaling anomalies phenomenologically, in the presence of a second length $\xi' \propto \delta^{-\nu'}$ in the VBS state, we propose that Eq. 1 should be replaced by the form

$$A(g, L) = L^{-\tilde{\kappa}/\tilde{\nu}} f(\delta L^{1/\nu}, \delta L^{1/\nu'}, L^{-\omega}) \quad (2)$$

where, unlike what was assumed in the past, $\tilde{\nu}$ is not necessarily the same as the exponent ν , which governs the behavior of most observables in the thermodynamic limit. Instead, we show that the criticality in the J - Q model generically demands that $\tilde{\nu} = \nu'$.

First, assume $\tilde{\nu} = \nu$. The correct thermodynamic limit with $\kappa = z\nu$ for ρ_s can then be obtained from Eq. 2 if $f(x, y, L^{-\omega}) \propto x^{z\nu}$ for large $x = \delta L^{1/\nu}$, $y = \delta L^{1/\nu'}$, and, as before, $\rho_s(\delta = 0, L) \propto L^{-z}$. This can also be expressed by using a scaling function in which the second argument is the ratio of the two lengths,

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$\tilde{f}(\delta L^{1/\nu}, L^{1/\nu'-1/\nu}, L^{-\omega})$. If $\tilde{f}(\delta = 0)$ is constant when $L \rightarrow \infty$, then $L^{1/\nu'-1/\nu}$ acts like just another irrelevant field, as in the standard scenario for dangerously irrelevant perturbations in classical clock models (31). Our proposal is a different large- L limit of Eq. 2, controlled by $y = \delta L^{1/\nu'}$, which leads to concrete predictions of scaling anomalies. In the case of the stiffness, the correct thermodynamic limit is obtained with $\tilde{\nu} = \nu'$ and $\kappa = \kappa\nu$ if $f(x, y, L^{-\omega}) \propto y^{\nu'}$ for large L . Then $\rho_s(\delta = 0) \propto L^{-2\nu/\nu'}$, which we can also obtain with $\tilde{\nu} = \nu$ and $\tilde{f} \propto L^{\kappa(1-\nu/\nu')}$ for $\delta \rightarrow 0$. A function $\tilde{f}$ behaving as a power of L was implicitly suggested in (19), though with no specific form.

This alternative scaling behavior corresponds to $\xi \propto (\xi')^{1/\nu'}$ saturating at $\xi \propto L^{1/\nu'}$ when $\xi' \rightarrow L$ upon approaching the critical point, in contrast to the standard scenario in which ξ grows until it also reaches L (32). The criticality at distances $r < L^{1/\nu'}$ is conventional, whereas $r > L^{1/\nu'}$ is governed by the unconventional power laws. Different behaviors for $r \ll L$ and $r \approx L$ were observed in a recent loop-model study (24), and a dangerously irrelevant field was proposed as a possible explanation, but with no quantitative predictions of the kind offered by our approach. The anomalous scaling law controlled by ν/ν' , which we confirm numerically below, is an unexpected feature of DQC physics and may also apply to other systems with two divergent lengths.

The J - Q model (15) for spins $S = 1/2$ is defined using singlet projectors ($P_{ij} = 1/4 - S_i \cdot S_j$) as

$$H = -J \sum_{\langle ij \rangle} P_{ij} - Q \sum_{\langle ijkl \rangle} P_{ij} P_{kl} \quad (3)$$

where $\langle ij \rangle$ denotes nearest-neighbor sites on a periodic square lattice with L^2 sites, and ij and kl in $\langle ijkl \rangle$ form the horizontal and vertical edges of 2×2 plaquettes. The Hamiltonian H has all symmetries of the square lattice, and the VBS ground state for $g = J/Q < g_c$ (with $g_c \approx 0.045$) is columnar, breaking the translational and 90° rotational symmetries spontaneously. The Néel state for $g > g_c$ breaks the spin rotation symmetry.

Although we have argued that the asymptotic $L \rightarrow \infty$ behavior when $\delta \neq 0$ in Eq. 2 is controlled by the second argument of f , the critical finite-size scaling close to $\delta = 0$ (when $\delta L^{1/\nu}$ is of order 1) can still be governed by the first argument (32). We will demonstrate that, depending on the quantity, either $\delta L^{1/\nu}$ or $\delta L^{1/\nu'}$ is the relevant argument, and, therefore, ν and ν' can be extracted using single-parameter scaling. We will first consider dimensionless quantities, corresponding to $\kappa = 0$ in Eq. 2, before testing the anomalous powers of L in other quantities.

If the effective one-parameter scaling holds close to g_c , then Eq. 2 implies that $A(g, L_1) = A(g, L_2)$ at some point g that we denote $g^*(L_1, L_2)$, and a crossing-point analysis (Fisher's phenomenological renormalization) can be performed (29). For a $\kappa = 0$ quantity, if $L_1 = L$ and $L_2 = rL$ with $r > 1$ being constant, a Taylor expansion of f shows that the crossing points $g^*(L)$ approach g_c as $g^*(L) - g_c \propto L^{-(1/\nu+\omega)}$, if ν is the relevant exponent (which we assume here for definiteness). $A^* =$

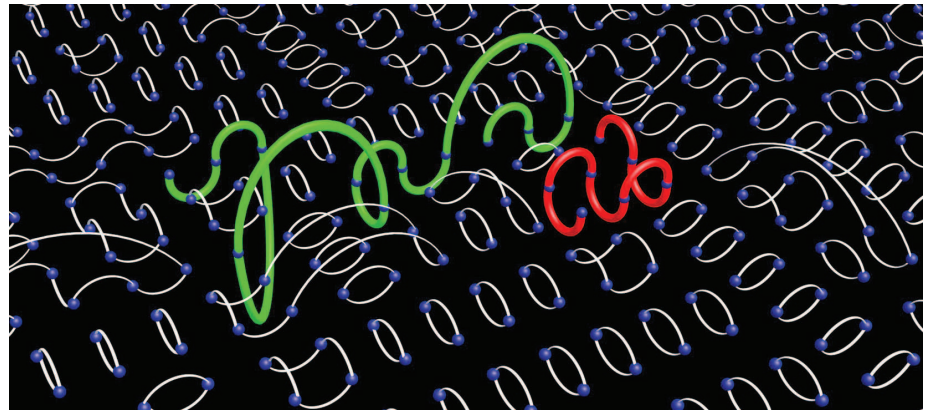


Fig. 1. Illustration of spinons. Shown is a QMC transition graph (33, 34) representing a sampled overlap $\langle \psi_{\text{left}} | \psi_{\text{right}} \rangle$ of $S = 1$ states with two strings (spinons, shown in red and green) in a background of valence-bond loops. Arches above and below the plane represent the states $|\psi_{\text{right}}\rangle$ and $|\psi_{\text{left}}\rangle$, respectively.

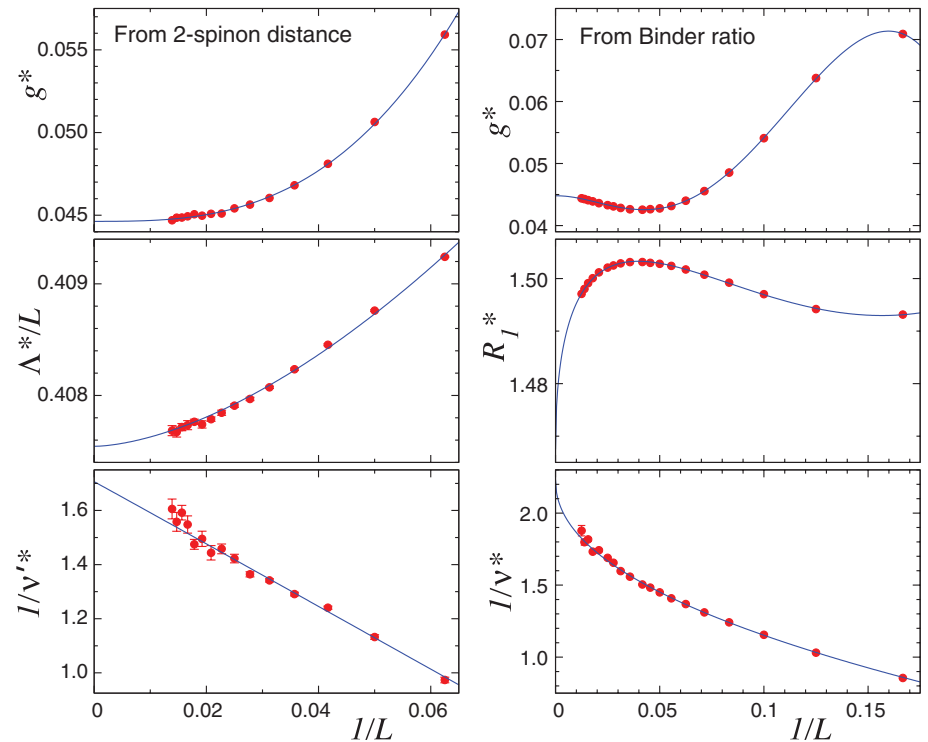


Fig. 2. ($L, 2L$) crossing-point analysis. The size of the spinon bound state and the Binder ratio were used to generate the left and right panels, respectively. The monotonic quantities were fitted with simple power-law corrections; two additional subleading corrections were included in the fits of the non-monotonic quantities.

$A(g^*)$ approaches its limit A_c as $A^*(L) - A_c \propto L^{-\omega}$, and it can also be shown that the quantity

$$\frac{1}{\nu^*(L)} = \frac{1}{\ln(r)} \ln \left(\frac{dA(g, rL)/dg}{dA(g, L)/dg} \right)_{g=g^*} \quad (4)$$

converges to $1/\nu$ at the rate $L^{-\omega}$. In practice, simulation data can be generated on a grid of points close to the crossing values, with polynomials used for interpolation and derivatives. We present details and tests of such a scheme for the Ising model in (32).

In the $S = 1$ sector, spinon physics can be studied with projector QMC simulations in a basis of valence bonds (singlet pairs) and two unpaired spins (33, 34). Previously, the size of the spinon bound state in the J - Q model was extrapolated to the thermodynamic limit (35), but the results were inconclusive as to the rate of divergence upon approaching the critical point. Here we consider the critical finite-size behavior. We define the size Λ of the spinon pair by using the strings connecting the unpaired spins in valence-bond simulations (Fig. 1) (32–34).

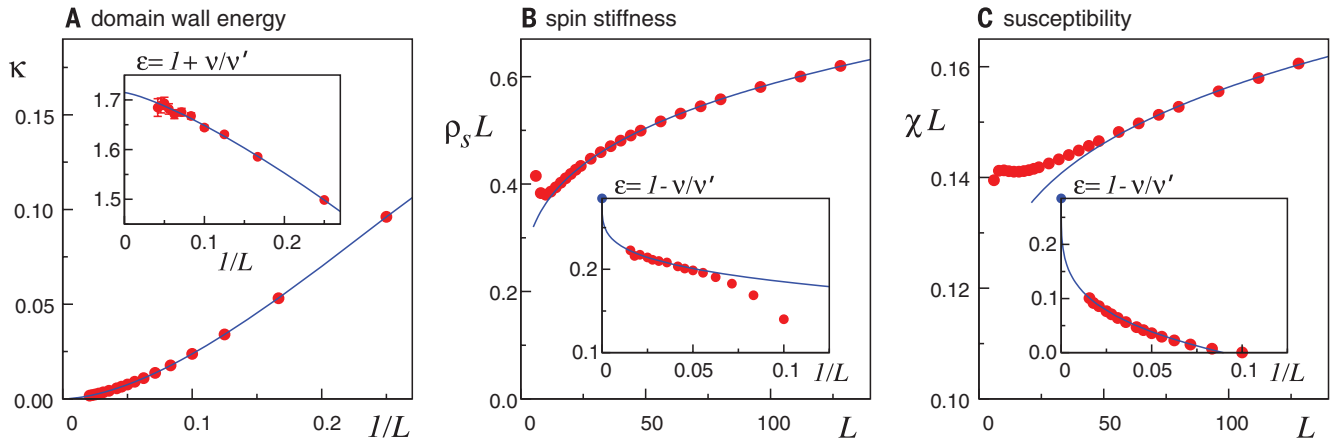


Fig. 3. Consistent anomalous critical scaling. Various quantities y are shown at $J/Q = 0.0447$. The insets show running exponents $\epsilon(L) = |\ln(y_L/y_{2L})|/\ln(2)$. **(A)** A fit of $\epsilon(L)$ gave $1 + v/v' = 1.715$ for $L \rightarrow \infty$ and a correction $\propto L^{-1.2}$. **(B and C)** Fixing the value of $1 - v/v'$ at 0.285, corrections $\propto L^{-\omega}$ with $\omega \approx 0.3$ were fitted to $\epsilon(L)$ for large L . The same values of v/v' and ω were used in the curves of the form $L^{1-v/v'}(a + bL^{-\omega})$ in the main graphs (where a and b are fitted constants).

If $\Lambda(g) \propto \xi'(g)$ when $L \rightarrow \infty$, then $\Lambda(g_c) \propto L$ follows from our proposed limit of Eq. 2. If Λ manifestly probes only the longer length scale in a finite system, which we will confirm below, then v' is the exponent controlling the crossing points of Λ/L . Data and fits are presented in Fig. 2 (left side). Unlike other quantities that have been used previously to extract the critical point (18), the drift of g^* with L is monotonic in this case, and the convergence is rapid. All $L \geq 16$ points are consistent with the expected power-law correction, with $1/v' + \omega \approx 3.0$ and $g_c = 0.04468(4)$, where the number in parenthesis indicates the statistical uncertainty (one standard deviation) in the preceding digit. The critical point agrees with earlier estimates (18). The scaled crossing value Λ^*/L also clearly converges, and a slope analysis according to Eq. 4 gives $v' = 0.585(18)$.

In Fig. 2 (right side), we show the analysis of a Binder ratio, defined with the z component of the sublattice magnetization m_{sc} as $R_1 = \langle m_{sc}^2 \rangle / \langle |m_{sc}|^2 \rangle$ and computed at $T = 1/L$ as in (18). In this case, the nonmonotonic behavior of the crossing points necessitates several scaling corrections, unless only the largest sizes are used. In either case, the $L \rightarrow \infty$ behavior of g^* is fully consistent with the g_c obtained from Λ/L . $R_1(g_c)$ has an uncertainty of over 1% because of the small value of the correction exponent, $\omega \approx 0.4$ to 0.5 . The slope estimator (Eq. 4) of the exponent $1/v$ is monotonic and requires only a single $L^{-\omega}$ correction, also with a small exponent $\omega \approx 0.45$. The extrapolated exponent $v = 0.446(8)$ is close to the value obtained recently for the loop model (24).

The above results support a nontrivial deconfinement process in which the size of the bound state diverges faster than the conventional correlation length. However, in the DQC theory, the fundamental longer length scale ξ' is the thickness of a VBS domain wall. It can be extracted from the domain-wall energy per unit length κ , which in the thermodynamic limit should scale as $\kappa \propto (\xi\xi')^{-1}$ (4). In (32), we re-derive this form using a two-length scaling ansatz and discuss simulations of domain walls in a 3D clock model

and the J - Q model. At criticality in the conventional scenario (exemplified by the clock model), both ξ and ξ' saturate at L and $\kappa \propto L^{-2}$. For the J - Q model with large L , we instead find $\kappa \propto L^{-a}$ with $a = 1.715(15)$ (Fig. 3A). Our interpretation of this unconventional scaling is that when ξ' saturates at L , ξ also stops growing and remains at $\xi \propto L^{v/v'}$. Thus, $\kappa \propto L^{-(1+v/v')}$ with $v/v' = a - 1 = 0.715(15)$, which agrees reasonably well with $v/v' = 0.76(3)$, obtained from the quantities in Fig. 2. The large error bar on the latter ratio leaves open the possibility that the spinon confinement exponent is between v and the domain-wall exponent v' (4).

We also calculated the critical spin stiffness ρ_s and the susceptibility $\chi(k = 2\pi/L)$ for the smallest wavenumber k at $T = 1/L$. Conventional quantum critical scaling (2) dictates that both quantities should decay with L as $1/L$. Instead, panels B and C in Fig. 3 demonstrate slower decays, with $L\rho_s$ and $L\chi$ being weakly divergent, as has also been found in earlier works (17–19, 21, 30). The unconventional limit of the scaling function in Eq. 2 requires $L\rho_s$ and $L\chi$ to diverge with L as $L^{1-v/v'}$. The behaviors are consistent with $v/v' \approx 0.715$, extracted from κ and a correction $\propto L^{-\omega}$ with a small ω (close to the correction for R_1 in Fig. 2). The mutually consistent scaling of the three quantities lends strong support to a type of criticality in which the magnetic properties are not decoupled from the longer VBS length scale ξ' for finite L . The results are incompatible with a first-order transition, where $\kappa \rightarrow \text{constant}$, $L\rho_s \rightarrow L$, and $L\chi \rightarrow L$.

We have shown that the effects of the larger divergent length scale ξ' at the Néel-VBS transition are more dramatic than those caused by standard dangerously irrelevant perturbations (31), and we therefore propose the term “super-dangerous” for this case. The universality class, in the sense of the normal critical exponents in the thermodynamic limit at $T = 0$, is not affected by such perturbations, but anomalous power laws of the system size appear generically in finite-size scaling. We have determined the value $v/v' \approx 0.72$

for the exponent ratio governing the anomalous scaling in the J - Q spin model.

Loop and dimer models exhibit similar scaling anomalies (24, 25), and it would be interesting to test the consistency between different quantities in these models, as we have done in this study. In simulations of the NCCP¹ action (21, 26, 27), one might not expect any effects related to the longer DQC length scale, because the monopoles responsible for the VBS condensation are not present in the continuum theory (3). Nevertheless, there could be some other super-dangerous operator present (24), perhaps related to lattice regularization.

The consequences of our findings carry over also to $T > 0$ quantum criticality in the thermodynamic limit, because $1/T$ is the thickness of an equivalent system in the path integral formulation (1, 2). Anomalous finite- T behaviors of the J - Q model have already been observed (18, 30). For instance, the spin correlation length at $T > 0$, which should be affected by deconfined spinons, grows more quickly than the normally expected form T^{-1} , and the susceptibility vanishes more slowly than T when $T \rightarrow 0$. The asymptotic forms $T^{-v/v'}$ and $T^{v/v'}$ can account for the respective behaviors (fig. S10). Thus, we find a strong rationale to revise the experimentally most important tenet of quantum criticality: the way that $T = 0$ scaling is related to power laws in T at $T > 0$. Our findings may apply to a wide range of strongly correlated quantum systems with more than one length scale and may help to resolve the mysteries that still surround scaling behaviors in materials such as high- T_c cuprate superconductors.

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SUPPLEMENTARY MATERIALS

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Supplementary Text
Figs. S1 to S10
References (36–41)

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BRAIN CONNECTIVITY

Task-free MRI predicts individual differences in brain activity during task performance

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When asked to perform the same task, different individuals exhibit markedly different patterns of brain activity. This variability is often attributed to volatile factors, such as task strategy or compliance. We propose that individual differences in brain responses are, to a large degree, inherent to the brain and can be predicted from task-independent measurements collected at rest. Using a large set of task conditions, spanning several behavioral domains, we train a simple model that relates task-independent measurements to task activity and evaluate the model by predicting task activation maps for unseen subjects using magnetic resonance imaging. Our model can accurately predict individual differences in brain activity and highlights a coupling between brain connectivity and function that can be captured at the level of individual subjects.

We all differ in how we perceive, think, and act. Our brains also differ in how they solve tasks. Understanding these individual differences in brain activity is an important goal in neuroscience, as it provides a route for linking brain and behavior.

Often, individual differences in brain activity induced by an experimental task are attributed to two possible factors. First, they may be accounted for by differences in gross brain morphology. The vast majority of brain-imaging studies rely on the spatial alignment of different brains (registration)

to account for between-subject discrepancies in gross anatomy (1). Second, subjects may use different strategies or cognitive processes that involve different brain circuits. Psychologists design their tasks with great care to limit this source of variability. Nevertheless, individual variations are seen in all behavioral domains (2–6). These between-subject differences are often treated as “noise” in imaging studies and discarded through the process of averaging individual responses (7). Indeed, it is thought that such individual differences are mainly explained by volatile factors related to the behavior.

We investigated the possibility that individual differences in brain activation are inherent features of individuals and, to a large degree, independent of volatile factors. We explored the extent to which individual differences in task-evoked brain activity can be predicted by differences in the functional connectivity of the brain, acquired in a magnetic resonance imaging (MRI) scanner while the subjects are at rest and not performing

any explicit task. We aimed to predict several task-evoked activity maps matching individual subject's maps in multiple behavioral domains based on a single task-free scan (i.e., unconstrained cognition during rest, with no explicit experimental task) of any given subject.

We designed a set of regression-based models that use task-independent features to predict individual task-evoked responses. Following the hypothesis that functional differentiation in the brain can be understood in terms of the underlying long-range brain connections and interactions (8, 9), we used predictors based on functional connectivity at rest (10). Brain networks, extracted from resting-state data sets, qualitatively resemble task-evoked networks at the group level (11). We therefore hypothesized that, using functional connectivity at rest, we could predict individual variations in task responses. We also used predictors encoding individual brain morphology (gross structure) and microstructure to yield a total of 107 predictors [see Materials and Methods in supplementary materials (SM)]. All of our predictors were based on imaging subjects at rest and were independent of any given task. The model was trained to map between the predictors and task activations in a cohort of subjects for each task, and subsequently, the trained model was applied to out-of-sample (unseen) subjects to predict their task activations (a leave-one-out approach, see Materials and Methods in SM for details). Throughout, we focused on predicting task activations on the cortex, although the approach can easily be extended to incorporate subcortical gray matter.

Our data were a subset (98 subjects) of the Human Connectome Project (HCP) database (12). HCP data were chosen for their inclusion of resting-state measurements, diffusion-weighted MRI, and structural MRI, as well as task-evoked data spanning several behavioral domains. We could therefore use the same set of task-free data to test predictions of several different tasks. The HCP task data include seven behavioral domains, and each task set comprises several statistical maps pertaining to different aspects of each task

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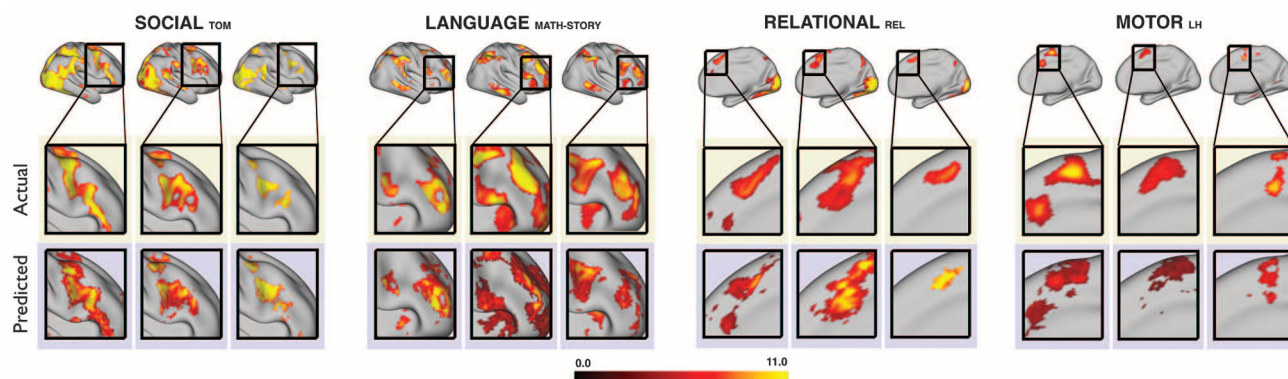


Fig. 1. Predicting individual variations in task maps. Figure shows actual and predicted thresholded task maps in three subjects and four different task contrasts. The model is able to capture striking variations between subjects in the shape, topology, and extent of their activation maps. Predictions and comparisons with actual maps for all behavioral domains and more subjects are shown in figs. S1 to S3 and movies S1 to S7.

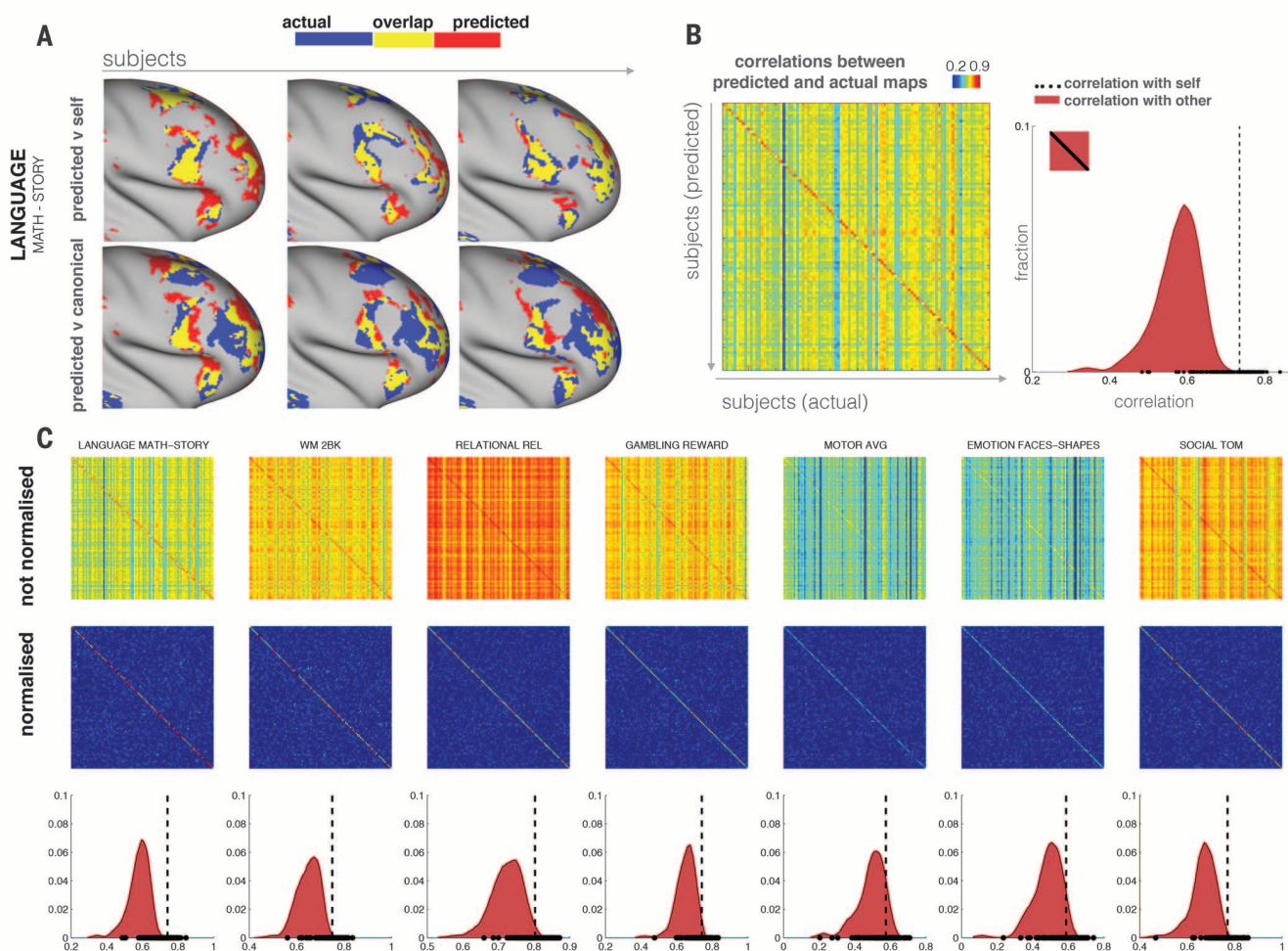


Fig. 2. Specificity of the individual predictions. A subject's predicted map is more similar to the subject's actual map than to the rest of the subjects. **(A)** Predicted maps (zoomed on the right frontal lobe) of the MATH-STORY contrast from the LANGUAGE task of three subjects are overlapped with their actual activation maps (top row). We also overlap the subjects' predictions with the activation map of the median subject (bottom row). Blue represents actual activation, red is the predicted activation, and yellow is the overlap. The maximum overlap is obtained when comparing a given subject's prediction with their own actual map. **(B)** Pearson correlation matrix between actual (columns) and predicted activations (rows). The correlation matrix is noticeably diagonal-dominant, which indicates that, on average, the model prediction for

any given subject is more similar to the subject's own map than to other subjects' maps. This is also shown as a histogram plot, where the extradiagonal elements of the correlation matrix (subject X versus subject Y) are compared with the diagonal elements (subject X versus subject X). The vertical dashed line corresponds to the median of the correlation coefficients along the diagonal. **(C)** Correlation matrices and histograms for six additional behavioral domains. When we normalized the rows and columns of the correlation matrices (which removes the mean and accounts for higher variability in actual than in predicted maps), the diagonal-dominance is even more prominent. In all cases, a Kolmogorov-Smirnov test between the two distributions (self versus other) gives a highly significant difference ($P < 10^{-10}$).

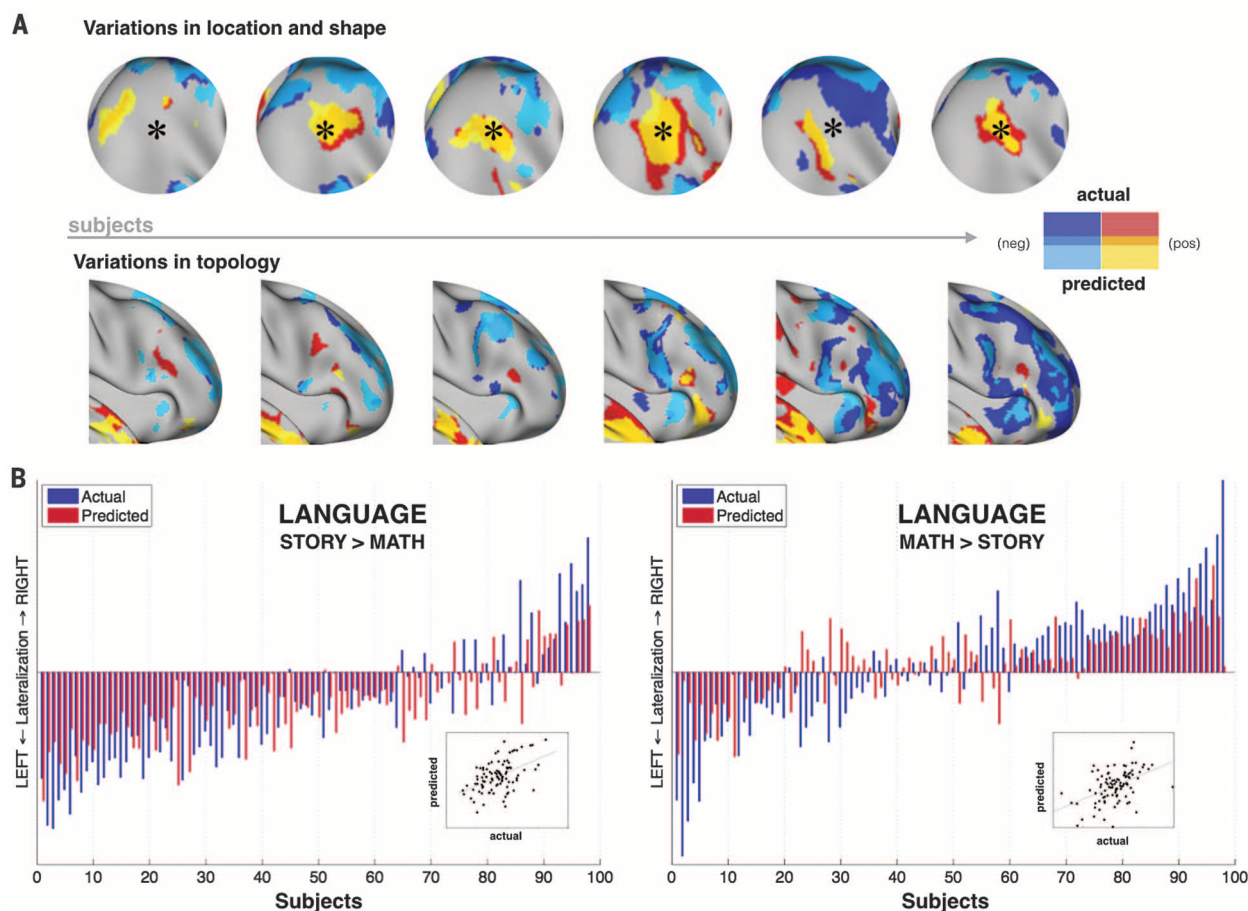


Fig. 3. Capturing qualitative and quantitative interindividual differences. (A) Variations in location, shape, and topology are predicted by the model (contrast: LANGUAGE MATH-STORY). (B) Peak Z scores were calculated for each hemisphere to examine how well the model can predict the amount of activation for each subject. A lateralization index (difference between right and left peak activation levels) is then calculated for each subject for both predicted and actual data and is shown as red and blue bars, respectively (LANGUAGE task). The model is able to predict individual subject's lateralization index for both contrasts, including the case where the majority of the subjects are left-lateralized. Statistical tests: MATH-STORY [correlation coefficient (r) = 0.47, $P < 10^{-6}$], STORY-MATH (r = 0.48, $P < 10^{-6}$).

[all tasks and derived parametric maps are described in (13)]. A total of 47 independent task maps (z scores) were available for each subject (see Materials and Methods in SM).

We aimed to predict not how subjects activate on average in a given task but how they differ (from each other) in their activation patterns. This aspect of the model is illustrated (Fig. 1) in four different task conditions (maps thresholded as described in Materials and Methods). The model could predict qualitative differences between subjects, in terms of shape, position, size, and topography of activations. Similar individual variations predicted across more subjects and all behavioral domains used by the HCP are shown in figs. S1 to S3 and movies S1 to S7. The model could precisely predict individual task variations across all behavioral domains, on the basis of a single task-free data set. To get an impression of how similar our predictions are to the actual task maps, we overlapped the predictions for three subjects with their actual activation maps (Fig. 2A) for the contrast MATH-STORY of the LANGUAGE task. For comparison, we also overlapped the same subjects with the actual activation map of a canonical (median) subject. The maximum

overlap was obtained when comparing a given subject's prediction with their actual map. This was despite our use of a leave-one-out approach: The predictive model for subject X had not seen the activation map of subject X during training, but it had seen the maps of all other subjects. Nevertheless, the prediction was more similar to the map it had not seen (subject X) than to the maps it had seen (all but subject X). The model did not learn what the activations for a given task looked like, but rather it learned how to map from the features (resting-state connectivity and morphology) to the task maps in individual subjects.

To quantitatively assess the performance of the model, we estimated the spatial correlation between the (unthresholded) predictions and actual maps for all pairs of subjects (Fig. 2, B and C). Each entry in the matrix is the Pearson correlation between the task map of one subject and the predicted map of another (off-diagonal) or the same (on-diagonal) subject. The correlation matrix is noticeably diagonal-dominant, which indicates that, on average, the model prediction for any given subject is more similar to the subject's own map than to other subjects' maps (see also the histograms shown in Fig. 2, B and C, comparing

on-diagonal with off-diagonal entries). Normalizing rows and columns of the correlation matrix (which removes the overall mean correlation and accounts for the fact that the actual maps are more variable than the predicted maps) shows the diagonal-dominance even more clearly (see also figs. S4 to S6, in which we show the same results for all contrast maps). The diagonal-dominance is apparent for all tasks and contrast maps, with the exception of one contrast map (GAMBLING REWARD). The reason is that activations for this contrast are restricted to subcortical gray matter, whereas our model only makes predictions for the cortex. This result, i.e., the diagonal dominance of the spatial correlation matrix, is nontrivial considering that our leave-one-out procedure ensures that, whenever a model is applied to predict a given subject, it has never seen that subject's task map during training. Yet, the prediction matches that subject's task data better than the subjects that it has seen during training. The model's ability to generalize beyond the training subjects has important implications. It can predict activations in individuals for which there are no available task data (e.g., patients who cannot perform the task).

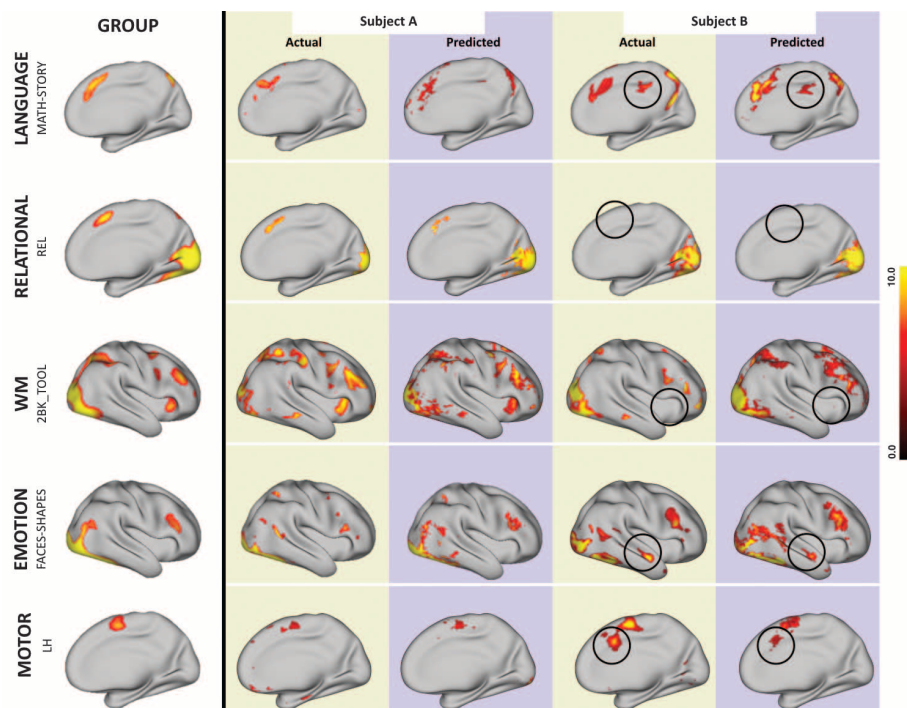


Fig. 4. Predictions in atypical subjects. The figure demonstrates the ability of our prediction model to detect intersubject variability when subjects differ from the group-averaged activation. In each row, the group activation for each behavioral domain is shown on the left, and the actual and predicted activations for two subjects are shown on the right. In each row, we show activations and predictions for one subject (A), whose activation is similar to the group activation, and another (B) who differs from it, as shown by the black circles. The model can capture the presence of clusters that are not active in the group (rows 1, 4, and 5), as well as the absence of clusters that are active in the group (rows 2 and 3). Note that subjects A and B are not the same pair of subjects across behavioral domains.

The model can precisely capture interindividual variability (table S3). There are different types of such variations. Functional brain areas in individual subjects can greatly vary in size, location, and shape (14). Primary visual cortex, for instance, can vary (across different subjects) by a factor of two or more in surface area (15). Such variability still allows one-to-one mapping of brain areas across subjects, provided that individual areas can be mapped in individual subjects. But another type of variation is topological variability, which for example means that different subjects may have different patterns of activity with no obvious one-to-one correspondence between subjects. Both types of variability (shape or size versus topological) can be captured by this model (Fig. 3A). Note that topological variability cannot be accounted for using spatial alignment between subjects, and thus, comparing or averaging subjects' activations when they have different topologies is an open problem. The fact that we can predict such variability from task-free data may enable new strategies for matching brain networks, as opposed to brain areas, across subjects.

Our model can also make predictions on the distribution of activity across different systems, such as in the case of hemispheric lateralization. Lateralization of function is seen commonly in the domain of language (16) but also in attentional processing (17). Our model can predict such differences across individuals in a language task, as

shown in Fig. 3B. We estimated a lateralization index defined as the difference between right hemisphere and left hemisphere peak Z (averaged over a 10-mm radius sphere around the predicted peak) from the predictions and the actual data. The model is able to precisely predict an individual subject's lateralization index. We also predicted two different contrasts from the same task (MATH-STORY and STORY-MATH), where the former tends to show an approximately equal distribution among left- and right-lateralized individuals, and the latter shows more left-lateralized subjects. The model can capture the lateralized language trend, even for the minority of right-lateralized subjects, despite the fact that, for the STORY-MATH contrast, it has been trained on subjects the majority of whom are left-lateralized.

The model could also predict atypical activation patterns. In Fig. 4, we show in five different behavioral domains examples of subjects that either do or do not conform to the pattern seen in the average subject. The model predicted activations in regions that were not activated on average. Conversely, the model correctly predicted the lack of activations in regions that were activated on average.

Overall, a simple set of models trained to learn a mapping between task-free and task-evoked maps could be used to predict individual differences in activation maps accurately across a

wide range of behavioral domains. The predicted maps matched even large individual variations in the spatial layout of brain activity, including position, shape, size, and topology of functional activations (e.g., whether the activity is localized or spread out, or whether it consists of a single region or multiple subregions). The model could also predict individual differences in the amount of activation in a given task from a task-free data set.

The model mainly used resting-state connectivity in addition to a few structural features capturing local morphology and microstructure (see Materials and Methods in SM). On average across all tasks, all features participated in the predictions except for subcortical connectivity features (excluding the cerebellum—see figs. S7 and S8). Although the structural features were exploited by the model (fig. S7), removing them did not affect the performance of the model (fig. S9), and using a model purely based on structural features abolishes interindividual variability in the predictions (fig. S10). This suggests that resting-state connectivity alone is sufficient to predict individual variability in task maps, independently from variations of morphology as captured by our handful of structural features. It is, however, possible that additional anatomical variability may also be indirectly captured by the resting-state data.

Why can we predict task-evoked activity from resting-state data? A good match between resting-state networks and task networks at the group level has been shown before (11, 18), where it has been argued that functional networks are continuously interacting with each other at rest, with the same functional hierarchy that is seen during action and cognition. Our model provides an explicit mapping from resting-state data to task maps that corroborates this assumption, but it is important to consider alternative explanations of our result. Intersubject alignment may be suboptimal when using anatomy alone. Our model is therefore possibly accounting for functional misalignment between subjects (19). However, the model not only tracks variation in the position and shape of functional areas but also captures variations in the topology of the brain networks activated in individual subjects and can predict activations of atypical subjects. Therefore, it is unlikely that the standard approach of aligning subjects while preserving the brain topology will be able to fix these types of misalignments. Our model can make quantitative predictions, in terms of the amount of activity that individual subjects display during a task. Such predictions are unlikely to arise from misalignment considerations. Resting-state data may provide a means for “calibration” of the blood oxygen-level-dependent (BOLD) signal (20). However, our predictors are based on measures of connectivity rather than raw BOLD signal strength per se [although signal-to-noise may still affect functional connectivity at rest (21)]. Determining what information in the resting-state signal is driving our predictions will be important for understanding its nature and potential. Because all the predictors that were used in this study are

based on scans acquired at rest, our model is blind to different strategies that are chosen by the participants in performing a given task. We refer to these features as “inherent,” but we acknowledge that these can be “structurally inherent” (related to brain organization and connectivity) or “functionally inherent” (related to the cognitive state of subjects during the resting-state scan).

The idea that brain connectivity can predict activation has previously been reported for different modalities (22), where diffusion MRI tractography was used (23) to measure connectivity. This study was limited to a specific cognitive task and a predefined anatomical region. More recently, resting-state connectivity has been shown to be predictive of subjects' identity, in a way similar to a fingerprint (24). Rather than simply identifying subjects, our goal was to predict the entire layout of brain activity for each subject. Moreover, we also aim to predict such layout of activity in a number of different cognitive domains, from a single task-free scan, including in subjects that show patterns of activation that are different from the group average (perhaps most strikingly in right-lateralized subjects when the majority of training subjects are left-lateralized).

There are important practical implications of the proposed framework in basic research and translational neuroscience. It provides a method for inferring multiple individualized functional localizers based on a single resting-state scan. Such a tool could be used to investigate in detail the response profiles of localized brain regions without the need to acquire often time-consuming task localizers. Such a tool, if generalizable beyond the young, healthy population that makes up the HCP database, could be used to investigate functional regions in subjects who cannot perform tasks, such as infants or paralyzed patients.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/352/6282/216/suppl/DC1

Materials and Methods

Figs. S1 to S23

Tables S1 to S3

Captions for Movies S1 to S7

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Movies S1 to S7

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POLITICAL SCIENCE

Durably reducing transphobia: A field experiment on door-to-door canvassing

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Existing research depicts intergroup prejudices as deeply ingrained, requiring intense intervention to lastingly reduce. Here, we show that a single approximately 10-minute conversation encouraging actively taking the perspective of others can markedly reduce prejudice for at least 3 months. We illustrate this potential with a door-to-door canvassing intervention in South Florida targeting antitransgender prejudice. Despite declines in homophobia, transphobia remains pervasive. For the intervention, 56 canvassers went door to door encouraging active perspective-taking with 501 voters at voters' doorsteps. A randomized trial found that these conversations substantially reduced transphobia, with decreases greater than Americans' average decrease in homophobia from 1998 to 2012. These effects persisted for 3 months, and both transgender and nontransgender canvassers were effective. The intervention also increased support for a nondiscrimination law, even after exposing voters to counterarguments.

Intergroup prejudice, defined broadly as negative attitudes about an outgroup, is a root cause of numerous adverse social, political, and health outcomes (1–3). Influential theories depict intergroup prejudices as deeply ingrained during childhood and highly resistant to change thereafter (4–6). Consistent with these theories, empirical research has found that durably reducing prejudice is challenging. Mass media interventions and other brief stimuli usually fail to reduce prejudiced attitudes (7) or have only temporary effects (8); lasting change ap-

pears to require intense intervention over months (9, 10). Rare are studies demonstrating prejudice-reduction interventions relatively brief in duration yet proven to have lasting effects (4).

Theories of active processing, however, suggest a method for even brief interventions to durably change attitudes. A recurring finding of laboratory studies is that brief messages can durably change individuals' attitudes when individuals engage in active, effortful, processing (known as “System 2” processing) of those messages (11). These studies, conducted on other topics, raise the possibility that brief interventions encouraging active consideration of counter-prejudicial thoughts could produce lasting changes in attitudes toward an outgroup. Perspective-taking, “imagining the world from another's vantage point,” is one

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thought process theorized to be especially cognitively active that has been shown to reduce prejudice in laboratory settings (12, 13). Together, these theories suggest that an intervention involving individuals to actively take an outgroup's perspective could durably reduce prejudice. Such an intervention is reported here.

The intervention we report attempted to reduce prejudice toward transgender people, those whose gender identity differs from the sex they were assigned at birth. Prejudice against transgender people (transphobia) is extremely pervasive in the United States, but research on the topic is limited and field studies are scarce (14). However, transphobia is important for at least two reasons. First, reducing transphobia is a recognized public health priority because it puts transgender people at up to 25 times greater risk of abuse, assault, and suicide (14). Attitudes toward transgender people are also of growing political importance. With tolerance toward lesbian, gay, and bisexual (LGB) people increasing, opponents of laws protecting LGB and transgender (LGBT) people from discrimination increasingly promulgate antitransgender stereotypes to build opposition to these laws. For example, a campaign in Houston, Texas, in November 2015 portrayed transgender women as “perverts” who sexually

assault young girls in women's restrooms, a strategy that observers believe contributed to voters' rejection of the city's nondiscrimination law (15).

Concerns that opponents of nondiscrimination laws would seek to exacerbate antitransgender prejudice in this manner motivated the intervention we report, door-to-door canvassing conversations in Miami, Florida. In December 2014, the Miami-Dade County Commission passed an ordinance protecting transgender people from discrimination in housing, employment, and public accommodations. Fearing a backlash that might increase transphobia, volunteers and staff from the Los Angeles LGBT Center (www.leadership-lab.org) and SAVE (a South Florida LGBT organization; www.save.lgbt) went door to door to have conversations with Miami-Dade voters.

For the intervention, canvassers first knocked on voters' doors unannounced. The one-time and uninvited nature of this contact method represents a substantial departure from most prejudice-reduction efforts previously studied, in which students or employees volunteer to repeatedly interact with teachers or supervisors (4). Canvassers asked to speak with the subject on their list and, once this person's identity was confirmed, identified themselves as volunteers from SAVE. The

canvassers then engaged in a series of strategies previously shown to facilitate active processing (11): Canvassers informed voters that they might face a decision about the issue (whether to vote to repeal the law protecting transgender people); canvassers asked voters to explain their views; and canvassers showed a video that presented arguments on both sides. Canvassers also defined the term “transgender” at this point and, if they were transgender themselves, noted this. The canvassers next attempted to encourage “analogic perspective-taking” (16). Canvassers first asked each voter to talk about a time when they themselves were judged negatively for being different. The canvassers then encouraged voters to see how their own experience offered a window into transgender people's experiences, hoping to facilitate voters' ability to take transgender people's perspectives. The intervention ended with another attempt to encourage active processing by asking voters to describe if and how the exercise changed their mind. The conversations lasted around 10 min on average. The supplementary materials provide further details.

Like other field studies investigating the effects of brief, psychologically informed interventions (17, 18), studying the effects of this intervention sheds light on the efficacy of theories of active

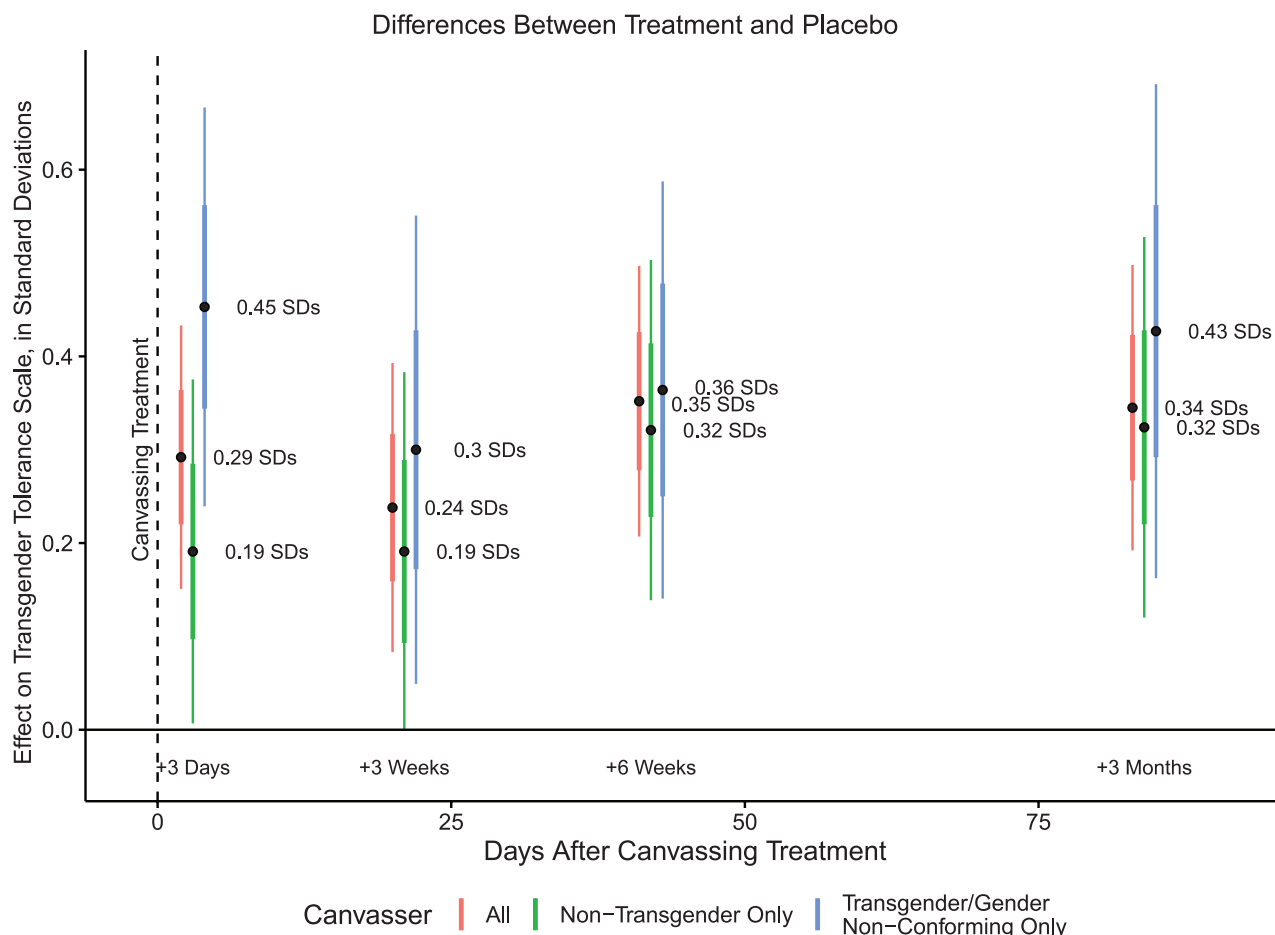


Fig. 1. Complier average causal effects on transgender tolerance scale. The 95% confidence intervals surround point estimates; the thicker lines represent one standard error. Both transgender and nontransgender canvassers produced large and lasting increases in tolerance.

processing and perspective-taking in a field setting. However, this focus on external validity means we cannot be certain that perspective-taking is responsible for any effects or that active processing is responsible for their duration; being primarily concerned with external validity and seeking to limit suspicion, we did not probe intervening processes or restrict the scope of the conversations as a laboratory study would. Nevertheless, as the supplementary materials describe, a majority of the training and conversations focused on

encouraging subjects to actively take transgender people's perspectives.

To measure the effects of these conversations, we conducted a randomized placebo-controlled experiment and parallel survey measurement. First, we recruited registered voters ($n = 68,378$) via mail for an ostensibly unrelated online baseline survey, presented as the first in a series of surveys. We next randomly assigned respondents to this baseline survey ($n = 1825$) to either a treatment group targeted with the intervention ($n = 913$) or

a placebo group targeted with a conversation about recycling ($n = 912$) (19). Random assignment to treatment or placebo was conducted at the household ($n = 1295$) level, such that subjects within the same household always had the same treatment assignment. Geographic clusters of respondents were also randomly assigned to have these conversations with canvassers who identified themselves to canvass leaders as transgender ($n = 15$) or as nontransgender ($n = 41$). The intervention then took place as described above;

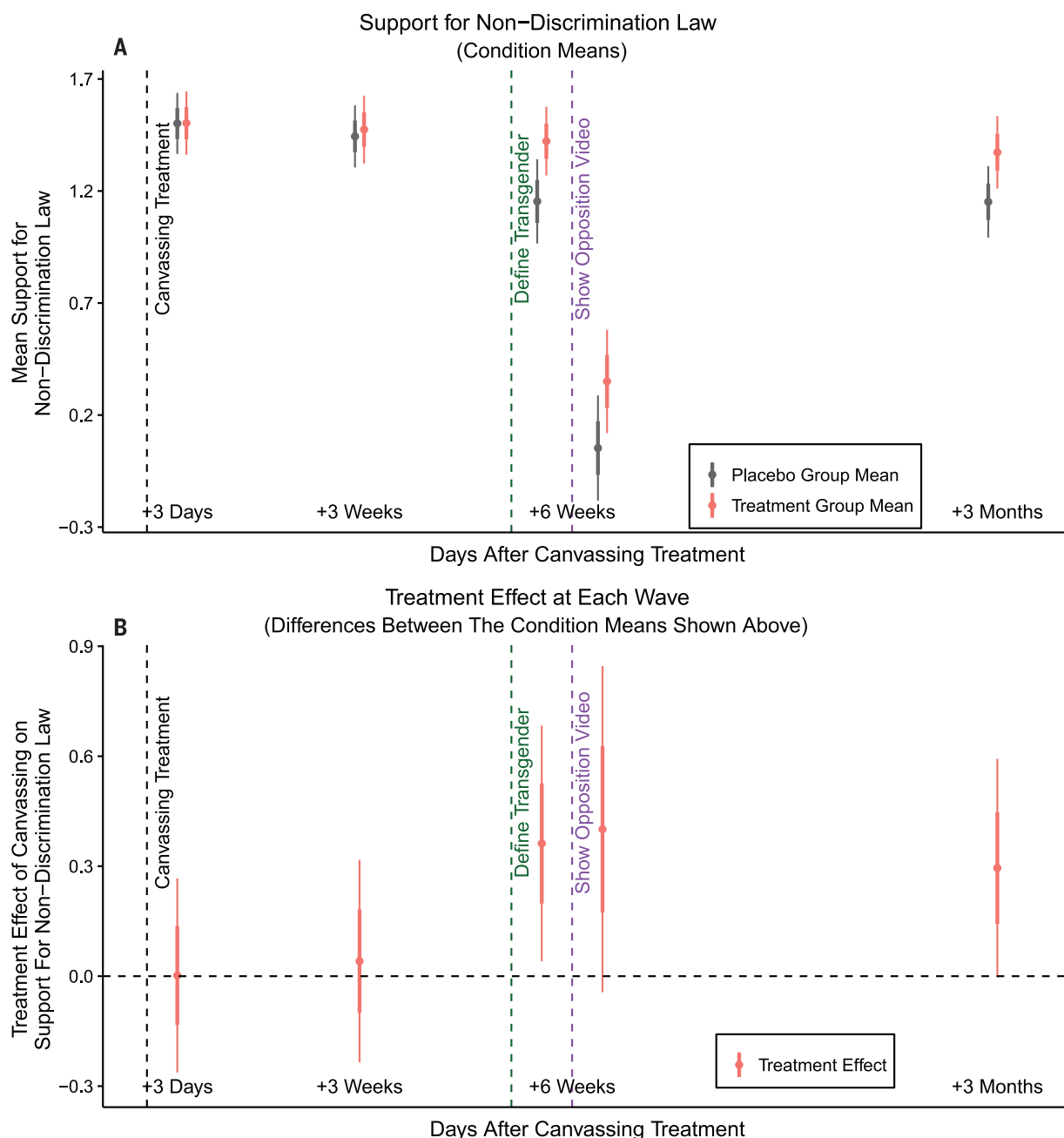


Fig. 2. Support for nondiscrimination law by survey wave. (A) Condition means. (B) Differences between these means. The 95% confidence intervals surround point estimates; the thicker lines represent one standard error. Standard errors are adjusted for clustering and pretreatment covariates. Defining “transgender” in the item wording revealed that placebo subjects were less supportive of protecting this group ($P < 0.05$, one-tailed). Next, all subjects were less supportive after viewing an opposing ad, but the effect of the conversations endured ($P < 0.05$, one-tailed). However, the ads’ impact dissipated, whereas the conversations’ effect endured ($P < 0.05$, one-tailed).

self-identified transgender canvassers revealed their identity to voters during these interactions. Finally, we recruited individuals who came to their doors in either condition ($n = 501$) to complete follow-up online surveys via email presented as a continuation of the baseline survey. These follow-up surveys began 3 days ($n = 429$), 3 weeks ($n = 399$), 6 weeks ($n = 401$), and 3 months ($n = 385$) after the intervention. See fig. S1 for an overview.

Two survey design features warrant note. First, although transgender rights have gained considerable media attention recently, many other derogatory terms are widely used to refer to this group, and we feared that many subjects would be unfamiliar with the term “transgender.” Therefore, survey item wordings generally eschewed the term “transgender.” One exception is an item about the law, presenting an issue that we will return to later. Second, in order to conceal its connection with the intervention, the survey was presented as a broad university-sponsored public opinion survey, and each wave included dozens of unrelated items, with only a few concerning transgender people. We carefully monitored responses for suspicion and found none.

The supplementary materials supply further recruitment, design, and estimation details, tests of design assumptions (tables S12 to S19), and representativeness assessments (table S20).

First, results indicate that the intervention was broadly successful at increasing acceptance of transgender people, as measured by an index of relevant items. Before the intervention, the treatment and placebo groups scored similarly on this index (see tables S13 to S17). After the intervention, the treatment group was considerably more accepting of transgender people than the placebo group ($t = 4.03$; $P < 0.001$). These effects are substantively large: These brief conversations increased positivity toward transgender people, as measured with a survey tool called a “feeling thermometer” (20, 21), by ~10 points, an amount larger than the average increase in positive affect toward gay men and lesbians among Americans between 1998 and 2012 (8.5 points) (see table S22). Figure 1 shows the point estimates at each wave and reveals that these effects persisted longitudinally: The treatment group remained more accepting in every follow-up survey (t tests; all P s < 0.01). (The point estimates are not strictly comparable over time because respondents and factor loadings change slightly; tables S1 to S4 show estimates for each item at each wave.) The intervention was also broadly effective: The effects are significant at the $P < 0.01$ level for both registered Democrats and registered Republicans (see table S6) and for those who began more and less supportive than average (t tests) (see table S8). Finally, conversations with transgender and nontransgender canvassers were both effective (t tests) (see table S5).

Second, these increases in acceptance of transgender people had ramifications for political attitudes: Treatment subjects were more supportive of the law protecting transgender people from discrimination than placebo subjects once the term “transgender” was defined for all subjects.

Figure 2A shows average support for the law, which was measured on -3 to +3 Likert scales, by condition and survey wave. Figure 2B shows the estimated treatment effect by survey wave. As Fig. 2 shows, there was no difference between the treatment and placebo groups’ support for a law protecting transgender people from discrimination in the 3-day and 3-week follow-up surveys. As we registered in a preanalysis plan before conducting the 6-week survey, we suspected that many placebo group subjects did not know what the term “transgender” meant (potentially being more familiar with other, derogatory terms for this group), making them unable to connect any antitransgender attitudes with this question about the law. However, the intervention informed treatment group subjects of the term’s definition, just as all subjects might learn the term’s definition were a political campaign ongoing. Likewise, views about transgender people might not have been focal for subjects when answering the question, whereas, during recent political campaigns, the inclusion of transgender people in nondiscrimination laws has been strongly emphasized (15). We therefore included a definition of the term “transgender” in the survey questions about the law, starting with the 6-week survey, clearly defining the term and highlighting transgender people’s inclusion in the law. As Fig. 2 shows, treatment subjects were 0.36 scale points more supportive of the law protecting this group than placebo subjects once the survey question defined the term “transgender” ($t = 2.20$; $P < 0.05$). This result is among the first experimental demonstrations of political attitudes exhibiting a long-lasting shift in response to persuasive communication (22).

One sign that attitude change is strong is that it persists longitudinally, which we have shown; another is that it withstands attack (17). Attack ads featuring antitransgender stereotypes are another common feature of political campaigns waged in advance of public votes on nondiscrimination laws (15). To examine whether support for the law would withstand such attacks, we showed subjects one of three such ads from recent political campaigns elsewhere, then immediately asked about the law again. Unsurprisingly, both groups were less supportive of the law immediately after viewing an attack ad. However, two patterns qualify the ads’ effect. First, the intervention’s effect withstood this attack: The canvassing intervention treatment group remained 0.40 scale points more supportive of the law than the placebo group ($t = 1.77$; $P < 0.05$, one-tailed). This result stands in contrast to the general pattern that competing messages mute the effect of political communication (23, 24) and suggests that the intervention would remain effective in a competitive political environment. Second, the ad’s effect faded, whereas the canvassing intervention’s effect persisted. When we asked the same question in the 3-month survey, both treatment and placebo subjects returned to their pre-ad attitudes. That the effect of the attack ads appeared to decay rapidly mirrors other research on political television advertising (22, 25). However, by contrast, the treatment effect of the canvassing con-

versations appeared to persist, because subjects in the canvassing treatment group remained 0.30 scale points more supportive than placebo subjects in the 3-month survey ($t = 1.94$; $P < 0.05$, one-tailed). These results suggest that the canvassing intervention’s effects were both lasting and politically relevant.

These findings have importance for a number of theoretical and applied questions. First, in light of influential theories that depict prejudiced attitudes as highly durable and resistant to change (4–7), it is surprising that brief personal interactions with strangers could markedly and enduringly reduce prejudice in a field setting. Rigorous field research has seldom documented brief interventions capable of producing large and lasting reductions in prejudice, leading the present results to represent a rare challenge to these theories.

The success of this approach was not obvious beforehand. Field settings present many barriers to perspective-taking, creating uncertainty about perspective-taking’s potential outside the laboratory and making field tests a research priority (12, 26). This setting presented further barriers still. Transgender people are extremely stigmatized (14), which could have led individuals to resist imagining transgender people’s perspective, especially when a transgender person was present (13, 26). That uninvited strangers could durably reduce prejudice when asking individuals to take transgender people’s perspective regardless of their own identity is thus particularly auspicious for these theories. However, whether active processing indeed moderates the persistence of perspective-taking’s effects is an open question better suited to laboratory research, as is whether “analogic” reflection on one’s own personal experiences of stigma facilitates taking the perspective of stigmatized groups.

On an applied level, the findings fill a void by providing prejudice-reduction advocates a feasible and proven strategy. A now-retracted article by LaCour and Green studying canvassing on marriage equality by the same organization, the Los Angeles LGBT Center, appeared in *Science* last year (27, 28). This article “provided a template” for gay rights advocates worldwide before its retraction (29). Several patterns in the present study’s data renew confidence that advocates could productively deploy the intervention strategy that we report. The intervention was effective among all prespecified subgroups, including political parties. Canvassers did not require extensive experience. Both first-time and experienced canvassers were effective, and most canvassers continued volunteering after the study concluded, indicating that organizations can develop activists who will work to widely deploy the strategy (30).

With this said, whether these theoretical and applied findings are limited in scope to negative attitudes toward transgender people is an open question that these findings cannot address; even though transgender people are widely stigmatized (14), attitudes toward them may be less entrenched than attitudes toward racial minorities, gays and lesbians, or other outgroups. The

experiment thus invites replication with other outgroups.

The intervention's durable effect on support for a nondiscrimination law also suggests optimism for the public sphere. Over the past century, political campaigns have increasingly relied on mass communication to reach voters (31). However, facing difficulty persuading a polarizing public with these strategies (22), campaigns increasingly eschew making the case for their positions and instead focus on rousing enthusiasm of voters who already agree with them (32). These shifts undermine basic aspirations for democratic discourse. However, these findings suggest that it may be in campaigns' own best interest to place renewed emphasis on a personal exchange of initially opposing views, even regarding controversial issues and across partisan lines.

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received compensation for this research. A preanalysis plan was filed after the data were collected but before they were analyzed with the treatment indicator, and revised preanalysis plans were also filed before the 3-week and 6-week surveys. These are available at the Evidence in Governance and Politics site (www.egap.org), ID 20150707AA. This research was approved by the University of California–Berkeley Committee for the Protection of Human Subjects, Protocol ID 2015-04-7508.

SUPPLEMENTARY MATERIALS

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CLIMATE CHANGE

Observational constraints on mixed-phase clouds imply higher climate sensitivity

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Global climate model (GCM) estimates of the equilibrium global mean surface temperature response to a doubling of atmospheric CO₂, measured by the equilibrium climate sensitivity (ECS), range from 2.0° to 4.6°C. Clouds are among the leading causes of this uncertainty. Here we show that the ECS can be up to 1.3°C higher in simulations where mixed-phase clouds consisting of ice crystals and supercooled liquid droplets are constrained by global satellite observations. The higher ECS estimates are directly linked to a weakened cloud-phase feedback arising from a decreased cloud glaciation rate in a warmer climate. We point out the need for realistic representations of the supercooled liquid fraction in mixed-phase clouds in GCMs, given the sensitivity of the ECS to the cloud-phase feedback.

Mixed-phase clouds, ubiquitous in Earth's atmosphere (1) at temperatures between 0° and –40°C, strongly influence Earth's radiation budget (2–4). For a fixed amount of cloud water, spherical liquid droplets tend to be smaller in size (5) and also to outnumber ice crystals, because ice nuclei (IN) are relatively scarce in Earth's atmosphere in comparison to cloud condensation nuclei (6). As a consequence, clouds that consist of a higher fraction of liquid are optically thicker and hence more reflective of sunlight. Here we refer to the fraction of supercooled liquid within a mixed-phase cloud at a particular isotherm as the supercooled liquid fraction (SLF).

It has recently been shown that SLFs are severely underestimated on a global scale in a multitude of global climate models (GCMs) (7, 8). This arises from two main causes. The first cause concerns the challenge of representing the microscopic nature of the various mixed-phase cloud

processes (9) that cannot be resolved at the typical spatial scales of GCMs. The Wegener-Bergeron-Findeisen (WBF) process for ice crystal growth is one such process that critically affects SLFs (8, 10–12). This process refers to the growth of ice crystals at the expense of surrounding supercooled liquid droplets in a mixed-phase cloud as a consequence of the lower saturation vapor pressure over ice relative to liquid. An ambient vapor pressure in between the saturation vapor pressures over liquid and ice is both a necessary and sufficient condition for the WBF process to occur in mixed-phase clouds (5). GCMs typically parameterize the WBF process by assuming homogeneous mixtures of supercooled liquid droplets and ice crystals within a model gridbox (10, 11). However, in situ observations suggest that such mixtures rarely exist in nature. Instead, pockets composed purely of either supercooled liquid droplets or ice crystals that are orders of magnitude smaller than the volume of a typical GCM gridbox are suggested to be the norm (13, 14). This implies that the WBF process may be too efficient in GCMs and is thus potentially a culprit for the underestimation of SLFs in GCMs. The multiple modes of ice nucleation (5), further complicated by the uncertainty associated with the efficiency

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of the various IN species (15), are additional factors contributing to the uncertainties in SLFs.

The second cause concerns the fact that mixed-phase clouds are poorly constrained because of the difficulty of obtaining observations of these clouds (16, 17). Although mixed-phase clouds are ubiquitous in Earth's mid- and high latitudes (1), in situ observations of these clouds are naturally limited by sparse spatial and temporal coverage. Technical difficulties in distinguishing liquid and ice particles of varying sizes, as well as artefacts associated with ice crystal shattering on probes (18), further complicate this matter. Satellite observations by NASA's Cloud-Aerosol Lidar with Orthogonal Polarization (CALIOP) instrument (19) offer an attractive alternative by providing global measurements of cloud thermodynamic phases since 2006.

To address the aforementioned issues, we constrained cloud phase in version 5.1 of the National Center for Atmospheric Research's Community Atmosphere Model (CAM5.1) (20) by using 79 months of observations obtained by CALIOP. CAM5.1 is a state-of-the-art GCM, used for climate studies worldwide, and is among the GCMs that severely underestimate SLFs over the entire globe relative to satellite observations (7, 8). To constrain CAM5.1-simulated SLFs to agree with observations, we adopted a quasi-Monte Carlo sampling approach to select 256 combinations of six cloud microphysical parameters (table S1), among which we included the WBF process time scale for the growth of ice crystals and the fraction of atmospheric aerosols active as IN (12). The WBF process is rendered less efficient in each case by retarding the time scale at which the process occurs. The default CAM5.1 ice nucleation scheme is replaced with one that has been developed based on in situ surface and aircraft observations to prognostically calculate the ice nucleating-particle concentration using the concentration of large dust aerosols (21). Out of the 256 simulations, two parameter combinations (table S1) that yield root mean

square errors of SLF < 0.050 across all isotherms were implemented into the fully coupled version of CAM5.1, CESM, version 1.0.6 (22), which includes interactive full-depth ocean, sea-ice, and land components. To benchmark these satellite-constrained simulations (hereafter referred to as CALIOP-SLF1 and CALIOP-SLF2), fully coupled simulations using the default model run without any modifications (henceforth referred to as control), as well as two more cases with unrealistically high and low SLFs meant to serve as the upper and lower bounds (henceforth referred to as high-SLF and low-SLF, respectively), were also run until the global net radiation budget at the top of the atmosphere was balanced with both present-day and doubled CO₂ concentrations, totaling 10 simulations altogether (table S2 and fig. S1). The simulated climate states are within realistic bounds (table S2). SLFs henceforth refer to those averaged over the past 50 years of simulation (the "mean state") with present-day CO₂ concentrations (the "initial state").

The mean extratropical (poleward of 30°) SLFs of the five cases are compared against each other in Fig. 1A, along with their ECS estimates in Fig. 1B. The correlation between SLF and ECS is evident upon comparison of the two figures [Pearson correlation coefficient (R) = 0.98, P = 0.0025]. The ECS estimates monotonically increase with increasing initial-state SLF, exhibiting a range extending from 3.9°C in low-SLF to 5.7°C in high-SLF. The ECS values of the other three cases exhibit a wide range of values in between these two unrealistic extremes. The control case has an ECS of 4.0°C, which is significantly lower than the satellite-constrained cases of 5.0° and 5.3°C, for CALIOP-SLF1 and CALIOP-SLF2, respectively. This result suggests that GCMs that underestimate SLFs may also be severely underestimating ECS. The upper bound of the observationally constrained estimates is greater than that of 30 GCMs participating in the Coupled Model Intercomparison Project (CMIP, version 5) (23). It should be noted, however, that the ECS

estimates of these 30 GCMs were computed using different methods and thus may not be directly comparable with the estimates presented here.

This increase in the ECS estimates is directly linked to a weakened negative cloud-phase feedback that affects shortwave (SW) more strongly than longwave (LW) radiation (2). In this feedback, an initial doubling of CO₂ concentrations causes the entire troposphere to deepen. In response, isotherms throughout the troposphere will shift upward in altitude relative to their location in the initial state. This implies that at any fixed altitude, there is a greater likelihood that the SLF of any mixed-phase cloud occupying that altitude will be higher than that of any pre-existing cloud at the same altitude in the initial state. Because mixed-phase clouds with higher SLFs are more reflective of SW radiation than those with lower SLFs, the enhanced reflection of SW radiation back to space counteracts the CO₂-induced warming. The cloud-phase feedback becomes less pronounced for mixed-phase clouds with higher initial-state SLFs (24). To illustrate this effect in the extreme, the feedback effectively vanishes for mixed-phase clouds with SLFs of unity, a scenario that is analogous to what occurs in high-SLF (Fig. 2, A and C). Here, the replacement of ice with liquid after CO₂ doubling only occurs between temperatures extending from ~ -30°C down to -40°C, which is approximately the temperature threshold for homogeneous freezing of liquid droplets. Furthermore, its strength is weakened by the fact that less cloud condensate exists at colder temperatures. As the negative cloud-phase feedback weakens, it is less effective at compensating for other processes that reduce cloud optical depth, which may include the drying of cloud layers by convective mixing (25) and rapid cloud adjustments to CO₂ (26). At the other extreme is low-SLF, which exhibits the strongest cloud-phase feedback (Fig. 2, B and D). Here, the feedback occurs throughout the heterogeneous freezing temperatures (0° to ~ -40°C). Although the cloud-phase feedback operates at all latitudes, it is strongest in the extratropics. At high latitudes, its effect on SW radiation is muted by the polar night.

The change in the gridbox-averaged liquid water path (Δ LWP), which measures the change in vertically integrated cloud liquid water content under global warming, monotonically decreases with increasing initial-state SLF (Fig. 1C). This is consistent with a weakening of the cloud-phase feedback. Although Δ LWP is positive in low-SLF, control, and CALIOP-SLF1 simulations, it eventually becomes negative in CALIOP-SLF2 and high-SLF, when the cloud-phase feedback has weakened to the point that other mechanisms cause the overall negative Δ LWP.

The mean extratropical net cloud optical depth feedback (λ_{τ}) quantifies the extent to which changes in the cloud optical depth amplify ($\lambda_{\tau} > 0$) or damp ($\lambda_{\tau} < 0$) the initial warming response to CO₂ doubling. The cloud-phase feedback is a primary but not the sole contributor to the cloud optical depth feedback by virtue of the vastly different optical depths of liquid and ice clouds

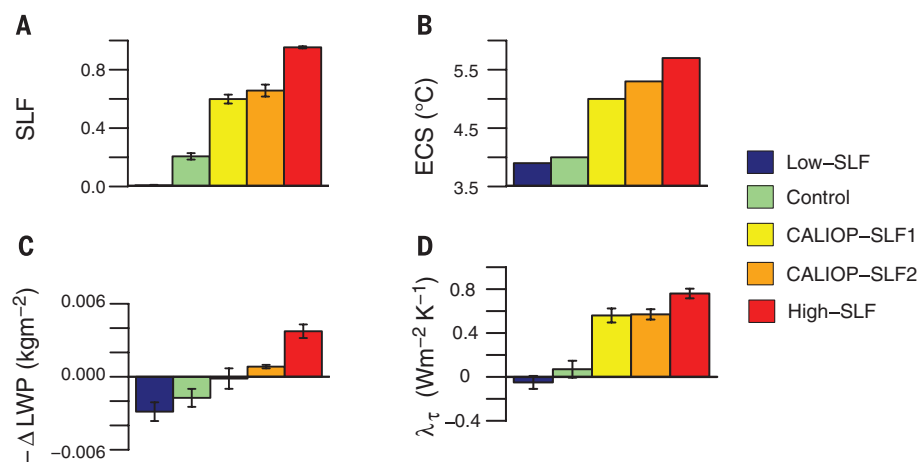


Fig. 1. The link between cloud thermodynamic phase partitioning and ECS. Error bars represent the 1 σ confidence interval. (A) The initial-state extratropical SLFs at the -10°C isotherm. (B) ECS estimates in response to CO₂ doubling. (C) Changes in global mean gridbox-average LWP. (D) Mean extratropical net cloud optical depth feedback, λ_{τ} .

(5). The monotonic increase in λ_τ is also consistent with a monotonic weakening of the cloud-phase feedback with increasing SLF (Fig. 1D). λ_τ is calculated based on the cloud radiative kernel method (27), using cloud fractions derived from the International Satellite Cloud Climatology Project satellite simulator (28, 29) implemented in CAM5.1. This method allows the total cloud feedback to be decomposed into individual contributions from the changes in cloud optical depth (λ_τ) and cloud-top pressure (i.e. height; λ_{CTP}) and

fraction (λ_{Amt}). Although the sum $\lambda_{CTP} + \lambda_{Amt}$ remains within 7% of that of the control across all cases, λ_τ monotonically increases with increasing SLF over a wide range primarily because of differences in reflected SW radiation (Fig. 3A). Regressions of cloud and noncloud feedbacks on ECS also strongly suggest that the cloud-phase feedback is driving the spread in ECS through its relation to λ_τ (fig. S2).

Global distributions of λ_τ elucidate the strength and locations over which the negative cloud-

phase feedback occurs. The negative cloud-phase feedback is strongest in low-SLF, and the high latitudes of both hemispheres show corresponding strongly negative λ_τ values (Fig. 3B). The weaker negative cloud-phase feedback in the control case exhibits less negative λ_τ values over the high latitudes (Fig. 3C). Eventually, the negative cloud-phase feedback is weakened to the extent that high-latitude λ_τ values become increasingly positive through the manifestation of other warming mechanisms in this order:

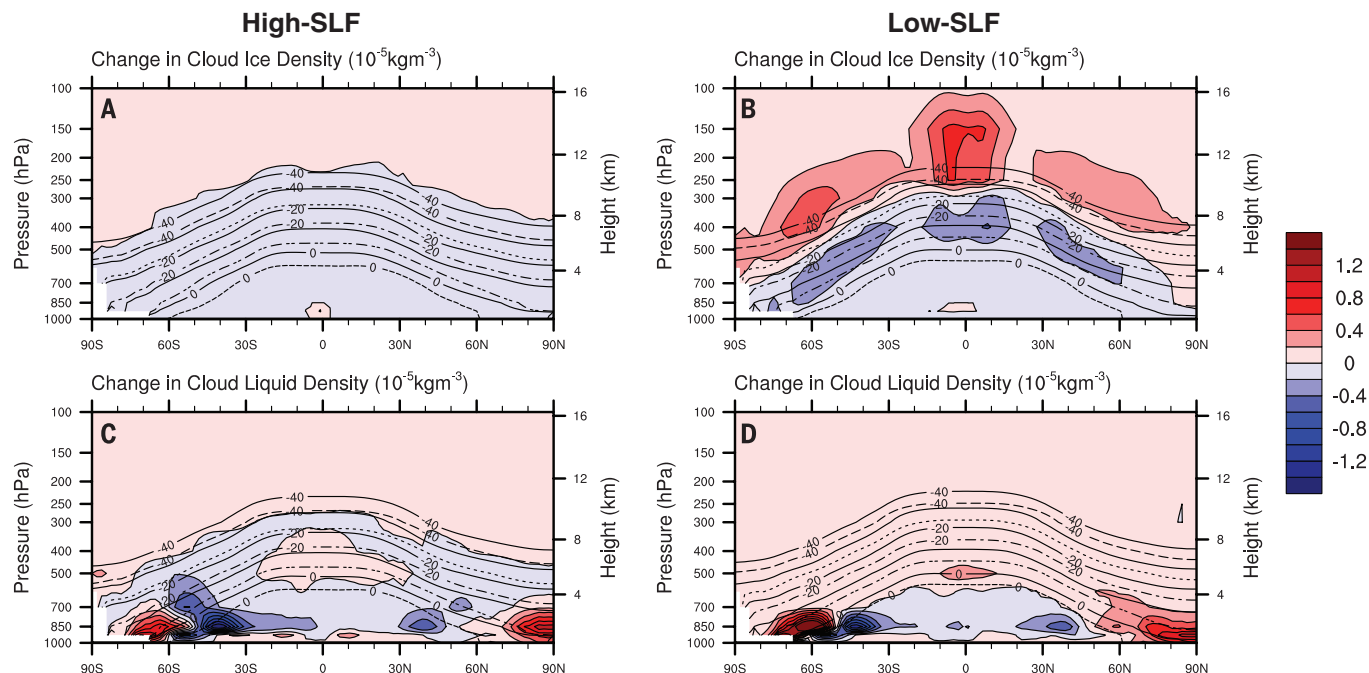


Fig. 2. Weakening of the cloud-phase feedback. Pressure-latitude cross-sections of zonal mean-state changes in gridbox-averaged (A and B) cloud ice and (C and D) cloud liquid densities in [(A) and (C)] high-SLF and [(B) and (D)] low-SLF in response to CO_2 doubling, exemplifying weakening of the cloud-phase feedback. Isotherms in the present-day CO_2 simulations are displayed as dashed lines; isotherms in the doubled CO_2 simulations are displayed as solid lines. The quantity of ice throughout the mixed-phase clouds in low-SLF decreases upon CO_2 doubling, while the quantity of liquid increases. The feedback still occurs in high-SLF, but it is substantially weakened, occurring only at the coldest isotherm.

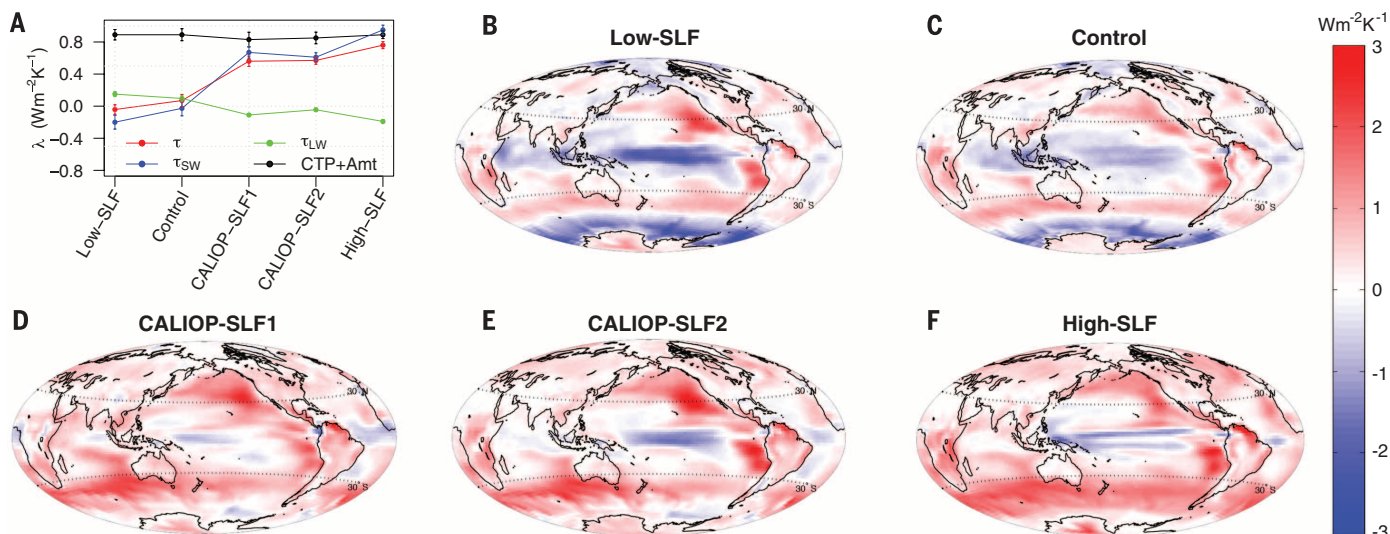


Fig. 3. Increase in the net cloud optical depth feedback parameter λ_τ with increasing initial mean-state SLF. Error bars represent the 1σ confidence interval. (A) Mean net extratropical λ_τ , decomposed into its SW and LW components, alongside the sum of the mean net extratropical λ_{CTP} and λ_{Amt} . (B to F) Global distributions of λ_τ .

CALIOP-SLF1 (Fig. 3D), CALIOP-SLF2 (Fig. 3E), and high-SLF (Fig. 3F). It is worth noting that the cloud-phase feedback is particularly sensitive to higher initial-state SLFs over the pristine Southern Ocean in the observationally constrained cases. Underestimates of SLFs relative to observations in this region (30) lead to an artificially stronger cloud-phase feedback that can ultimately lead to an underestimate of ECS. The negative λ_{τ} values over the tropical Pacific are attributed to the thickening of high clouds (27).

Global satellite observations of cloud thermodynamic phases have enabled us to show that unrealistically low SLFs common to a multitude of GCMs lead to a cloud-phase feedback that is too negative. This has important ramifications for ECS estimates. Should the low-SLF bias be eliminated in GCMs, the most likely range of ECS should shift to higher values. It should be noted that there are variations in the way in which microphysical processes are parameterized in GCMs. Thus, the method by which SLFs can be constrained in GCMs is not unique, and a repeat of this study in other GCMs may thus lead to other climate feedback responses that are not captured by the GCM used in this study. However, regardless of how SLFs are constrained, the SLF will affect the magnitude of the cloud-phase feedback and therefore GCMs' estimates of ECS. Looking forward, continued improvements of the accuracy of various observational methods, better understanding of mixed-phase cloud processes, and their improved representation in GCMs are all factors that are critical to improving the accuracy of ECS estimates.

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SUPPLEMENTARY MATERIALS

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CANCER BIOLOGY

MYC regulates the antitumor immune response through CD47 and PD-L1

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The *MYC* oncogene codes for a transcription factor that is overexpressed in many human cancers. Here we show that *MYC* regulates the expression of two immune checkpoint proteins on the tumor cell surface: the innate immune regulator CD47 (cluster of differentiation 47) and the adaptive immune checkpoint PD-L1 (programmed death–ligand 1). Suppression of *MYC* in mouse tumors and human tumor cells caused a reduction in the levels of CD47 and PD-L1 messenger RNA and protein. *MYC* was found to bind directly to the promoters of the *Cd47* and *Pd-l1* genes. *MYC* inactivation in mouse tumors down-regulated CD47 and PD-L1 expression and enhanced the antitumor immune response. In contrast, when *MYC* was inactivated in tumors with enforced expression of CD47 or PD-L1, the immune response was suppressed, and tumors continued to grow. Thus, *MYC* appears to initiate and maintain tumorigenesis, in part, through the modulation of immune regulatory molecules.

The transcription factor *MYC* regulates the expression of a multitude of gene products involved in cell proliferation, growth, self-renewal, differentiation, and apoptosis (1–4). The *MYC* gene is genetically activated and overexpressed in many human cancers (1–4), and this overexpression has been causally linked to tumorigenesis (5, 6). Studies involving inducible transgenic mouse models have shown that growth of *MyC*-induced tumors is dependent on continuous expression of *MYC* (1–4, 7–10). For example, in the tetracycline-off mouse model (where *Myc* expression can be turned off by the addition of tetracycline or doxycycline), tumors grow only when *Myc* is “on.” When *Myc* is turned “off,” tumors regress.

In mouse models, *MYC* inactivation results in tumor regression through the induction of proliferative arrest and apoptosis (1–3, 7, 8, 10–12). We have demonstrated that complete tumor clearance that occurs after the inactivation of oncogenes, including *Myc*, requires the recruitment of CD4⁺ T cells and the secretion of thrombospondin-1 (13, 14). Hence, a host-dependent immune response is required for sustained tumor regression. However, the mechanism by which oncogene inactivation elicits this immune response is unknown.

The host immune system generally serves as a barrier against tumor formation (15). Activation of the immune response can contribute to tumor regression (13, 16, 17) through both adaptive and innate immune effectors (18–20). Programmed death–ligand 1 (or PD-L1, also known as CD274 and B7-H1) sends a critical “don’t find me” signal to the adaptive immune system (21–23), whereas CD47 (cluster of differentiation 47) sends a critical “don’t eat me” signal to the innate immune system and acts as a regulator of the adaptive immune response (24, 25) (fig. S1A). These and similar molecules are often overexpressed on human tumors (22, 25). Therapeutic suppression of PD-L1

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and other immune checkpoint molecules elicits an immune response against tumors. Recently, this strategy has been applied in clinical practice, with very encouraging results (26–29).

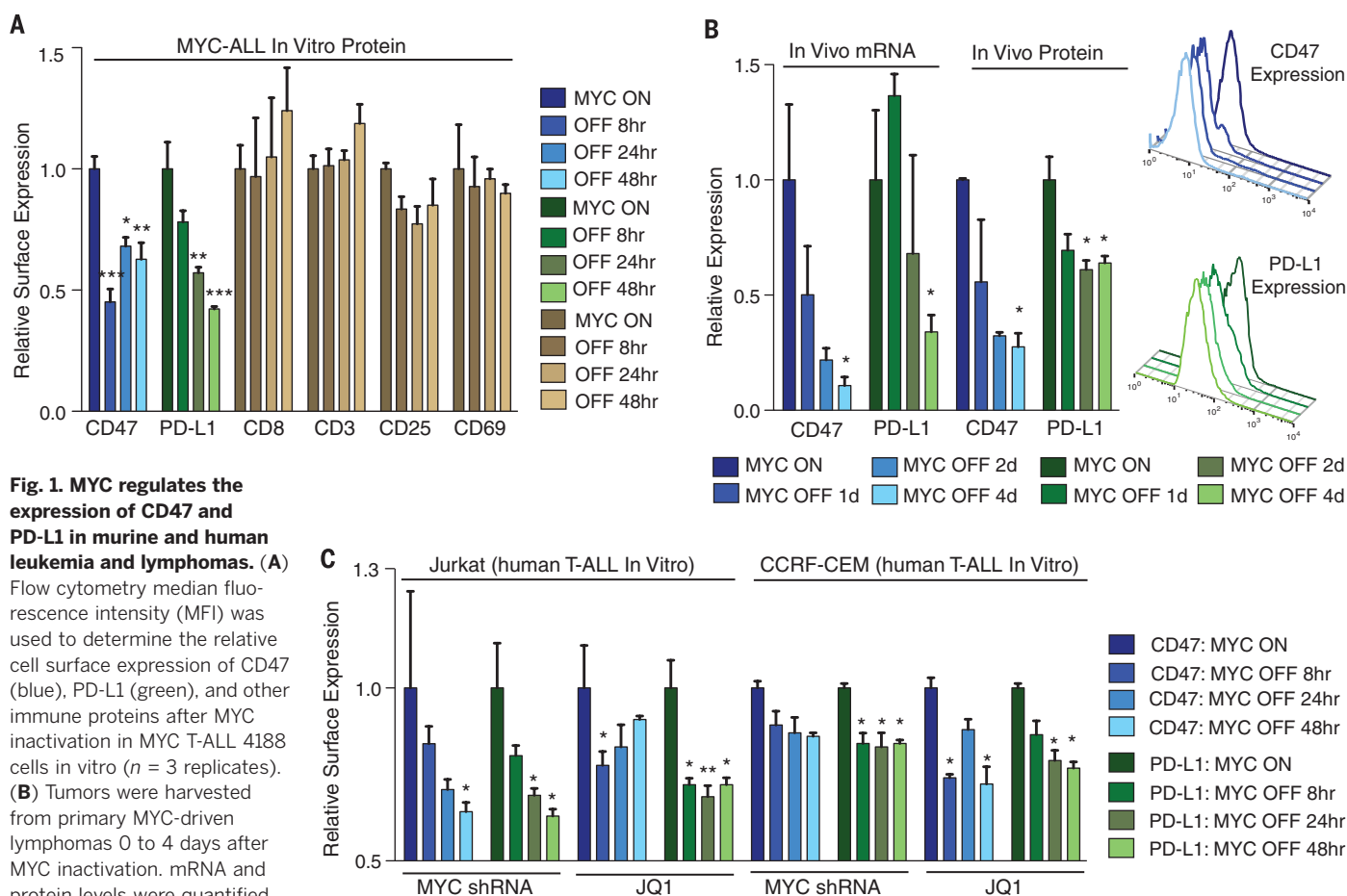
To explore whether and how *MYC* regulates the antitumor response, we examined its effect on the expression of CD47 and PD-L1 in the Tet-off transgenic mouse model of *MYC*-induced T cell acute lymphoblastic leukemia (*MYC* T-ALL). When *MYC* was turned on, both CD47 and PD-L1 were expressed. However, in vitro or in vivo *My*c inactivation resulted in a rapid down-regulation of CD47 and PD-L1—both at the mRNA level, as detected by quantitative real-time polymerase chain reaction (qPCR), and at the protein level, as detected by flow cytometry (Fig. 1, A and B) and immunofluorescence (fig. S1B). Expression of other immune-related surface receptors was not affected by *MYC* inactivation (Fig. 1A). Consistent with these observations, suppression of *MYC* expression in the human T-ALL cell lines CCRF-CEM and Jurkat, either by treatment with a *MYC*-targeting short hairpin RNA (shRNA) (fig. S2A) or with the BET (bromodomain and extraterminal) inhibitor JQ1 (30), reduced the expression of CD47 and PD-L1 (Fig. 1C). Treatment of *MYC* T-ALL cells with the chemotherapeutic drugs prednisone,

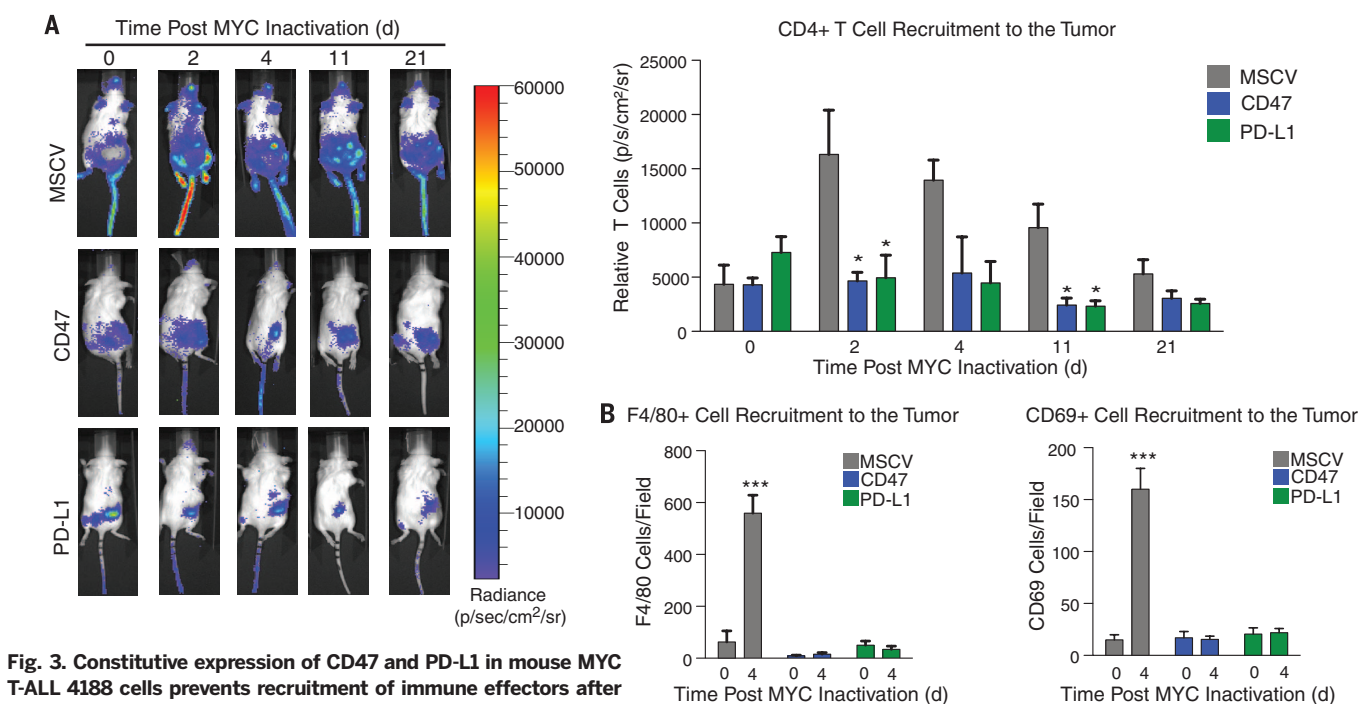
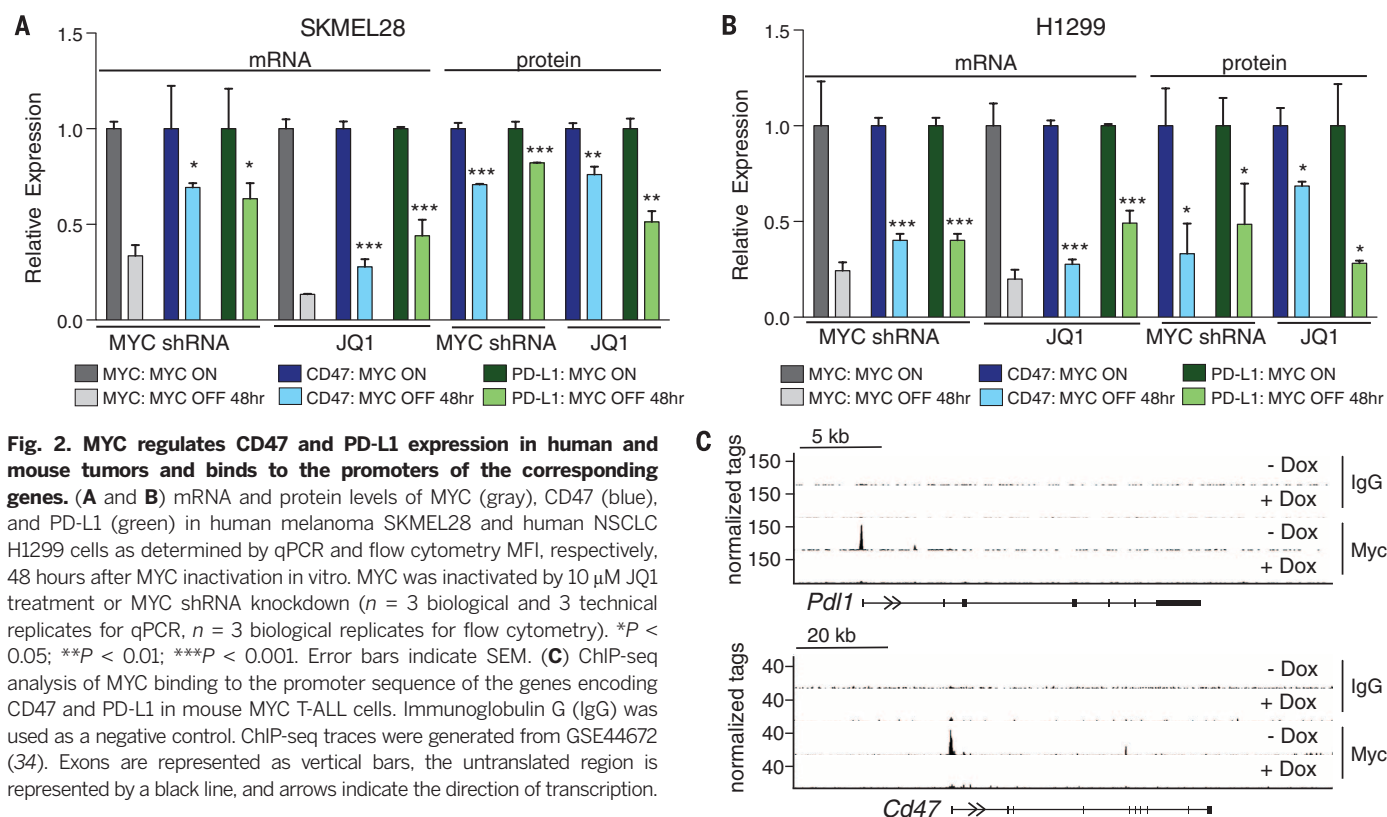
cytotoxin, cisplatin, or vincristine, resulted in tumor cell death. However, CD47 and PD-L1 were either unaffected or showed increased expression (fig. S3A), and there was no effect on the expression of CD3, CD8, CD25, or CD69 (fig. S3, B to E).

We next investigated the effect of *MYC* inactivation on CD47 and PD-L1 in mouse and human solid tumors. In a Tet-off transgenic mouse model of hepatocellular carcinoma (HCC) (3), inhibition of *MYC* expression resulted in decreased levels of CD47 and PD-L1 protein (fig. S4, A and B) and mRNA (fig. S4B); expression of the two proteins was not affected by cisplatin treatment (fig. S4A). In the human HCC cell line HEPG2, shRNA knockdown of *MYC* caused a reduction in the levels of both CD47 and PD-L1 mRNA (fig. S4C). We also investigated the relationship between *MYC* expression and CD47 and PD-L1 expression in the human melanoma cell line SKMEL28 (Fig. 2A) and the human non-small cell lung cancer (NSCLC) cell line H1299 (Fig. 2B), as these cells represent tumor types that are often treated with immune checkpoint inhibitors in the clinic (31). We found that *MYC* shRNA knockdown and *MYC* functional suppression by JQ1 reduced the expression of CD47 and PD-L1 mRNA and protein as measured by qPCR and flow cytometry, respectively.

In additional experiments, we found that *MYC* shRNA knockdown (fig. S2B) or JQ1 treatment of four independent primary human T-ALL samples reduced both CD47 and PD-L1 cell surface expression (fig. S5). Cisplatin treatment increased CD47 and PD-L1 expression, whereas CD8 expression was unaffected by the treatments (fig. S5). Last, we examined publicly available gene expression data derived from human primary tumors. Notably, in human HCC, renal cell carcinoma, and colorectal carcinoma, *MYC* expression significantly correlated with the expression of both CD47 and PD-L1 (fig. S6). Thus, *MYC* regulates CD47 and PD-L1 expression in multiple human tumor types.

MYC can act as a general transcriptional amplifier (that is, it can generally increase expression of many genes rather than specific target genes), but dosage-dependent specific effects have been reported (32–36). We analyzed chromatin immunoprecipitation sequencing (ChIP-seq) data from mouse *MYC* T-ALL cells (34) and the human B cell line P493-6 (37, 38) and found high levels of *MYC* bound to the promoter regions of the genes coding for CD47 and PD-L1 (Fig. 2C and figs. S7 and S8). In contrast, we observed that both *MYC* T-ALL (fig. S7) and P493-6 (fig. S8) cells with high levels of *MYC* had lower, often nonspecific,





or constitutive CD47- or PD-L1-expressing (colored) tumors before or 4 days after MYC inactivation, by immunohistochemistry using markers for macrophages (F4/80) and activated T cells (CD69). Tumor cells were transplanted into wild-type (WT) FVB hosts. Administration of Dox to inactivate MYC in established tumors occurred on day 0. The y axis denotes the number of positively-staining cells per field. For representative images, see fig. S13. Data represent mean $\pm$ SEM derived from measurements of three independent tumors and three measurements per tumor. * $P < 0.05$; *** $P < 0.001$.

binding to the promoters of other cell surface immune molecules such as CD8a and CD25. Oncogenic levels of MYC bound the CD47 and PD-L1 gene promoters in human osteosarcoma U2OS cells, whereas low levels of MYC did not (fig. S9). In a nuclear run-on assay with P493-6 cells, MYC induced expression of the *CD47* gene, along with other well-known target genes such as *PDK1*, *CHEK1*, *CDK2*, *LDHA*, and *ODC1* (fig. S10, A and B). PD-L1 expression was too low to measure changes in this experiment. Thus, we conclude that MYC binds to the promoters and directly regulates the expression of the *CD47* and *PD-L1* genes. An alternative but not mutually exclusive possibility is that MYC suppression acutely affects CD47 and PD-L1 surface protein expression by reducing the half-lives of the two proteins. However, we did not observe the increased turnover of CD47 or PD-L1 proteins compared with other immune surface proteins in mouse MYC T-ALL cells when we inhibited protein synthesis by cycloheximide treatment (fig. S11).

We have shown previously that MYC inactivation in mouse tumor models results in recruit-

ment of immune cells to the tumors (13). To investigate the role of CD47 and PD-L1 in this process, we engineered MYC T-ALL 4188 cells to constitutively express CD47 or PD-L1 (fig. S12A). In this overexpression system, CD47 and PD-L1 mRNA levels were unaffected by MYC inactivation (fig. S12B). The recruitment of luciferase-labeled CD4⁺ T cells (Fig. 3A), CD69⁺-activated T cells, and F4/80⁺ macrophages (Fig. 3B and fig. S13) after MYC inactivation was suppressed when CD47 and PD-L1 were constitutively expressed by the tumor cells. Expression of CD47 or PD-L1 prevented the sustained tumor regression that has been observed upon MYC inactivation (Fig. 4A), without affecting MYC expression (Fig. 4B). Enforced expression of CD47 or PD-L1 increased minimal residual disease (tumor cells remaining), resulting in tumor recurrence (Fig. 4, C and D). Conversely, shRNA knockdown of CD47 or PD-L1 prevented the growth of MYC T-ALL cells in vivo (fig. S14).

Inactivation of MYC induces tumor regression through both cell-autonomous mechanisms, including proliferative arrest and induction of apop-

toxis, and host-dependent mechanisms, such as inhibition of tumor angiogenesis and induction of tumor cell senescence (1–4, 13). We investigated the effect of enforced expression of CD47 or PD-L1 on these mechanisms and found that CD47 or PD-L1 expression prevented the shutdown of angiogenesis after MYC inactivation, as measured by the presence of CD31⁺ microvessels (Fig. 4E and fig. S15A) and expression of Ang2 and Tie2 (fig. S15C). The induction of tumor cell senescence, as measured by β -galactosidase (Fig. 4F and fig. S15B) and p15Ink4b and p19ARF levels (fig. S15D), was also affected, but we did not observe any effect on apoptosis or proliferation, as evaluated by annexin V and 7-AAD (fig. S16A), cleaved caspase 3 (fig. S16, B and D), and phospho-histone H3 (fig. S16, C and E). Therefore, the down-regulation of CD47 and PD-L1 appears to be required for the induction of sustained tumor regression, the shutdown of angiogenesis, and senescence induction promoted by MYC inactivation.

We conclude that MYC regulation of CD47 and PD-L1 expression has a direct role in the initiation

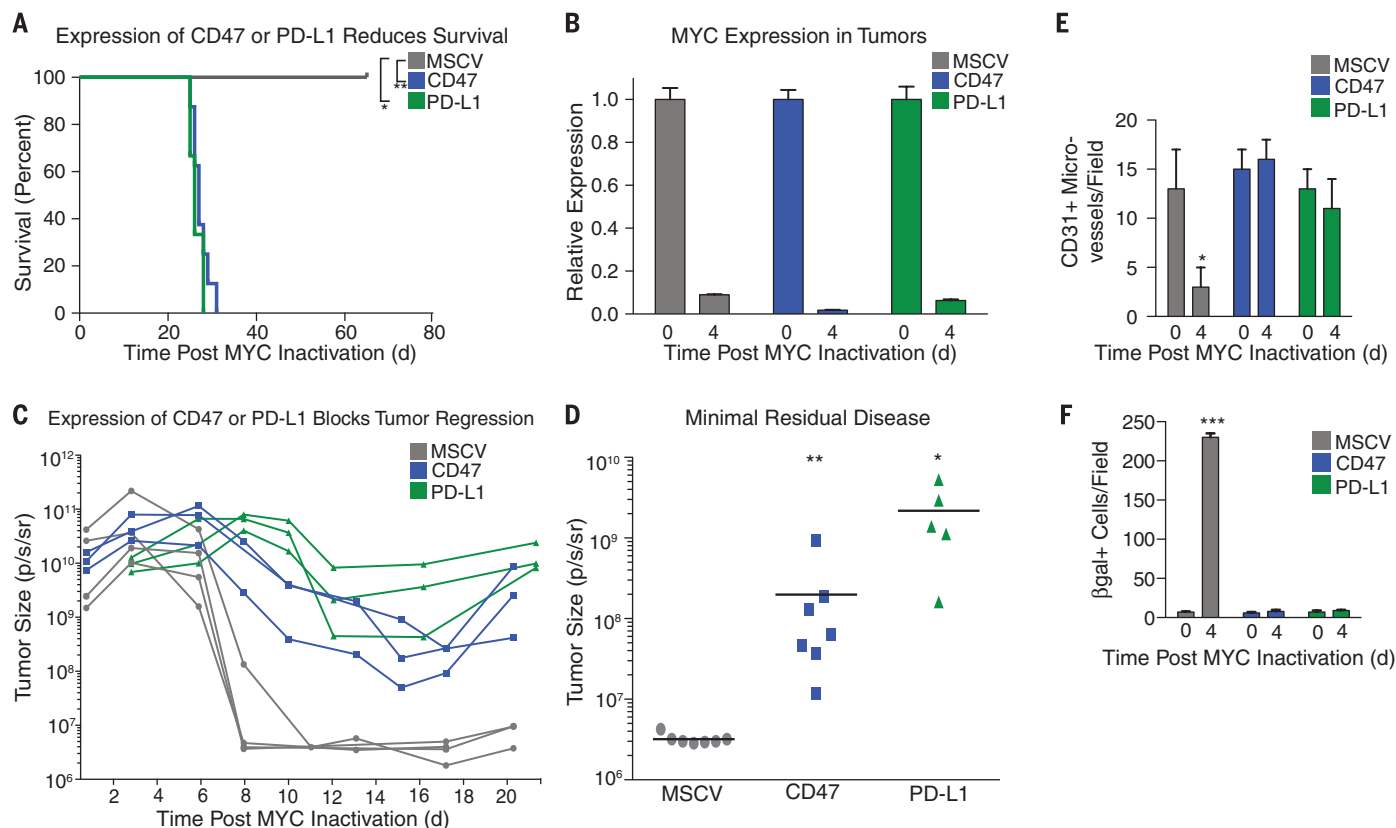


Fig. 4. Down-regulation of CD47 or PD-L1 is required for tumor regression, shutdown of angiogenesis, and induction of senescence upon MYC inactivation. (A) Survival after MYC inactivation of syngeneic FVB/N mice that had been transplanted with either MSCV control (gray), CD47-expressing (blue), or PD-L1-expressing (green) fLuc⁺ MYC T-ALL cells. MYC was inactivated when tumors reached 1.5 cm³ (day 0) ($n = 5$ tumors for control, $n = 10$ tumors for CD47, and $n = 5$ tumors for PD-L1). (B) MYC expression before (day 0) or after MYC inactivation (day 4). (C) Bioluminescence imaging measurement of tumor burden before and after MYC inactivation in control (gray), CD47-expressing (blue), and PD-L1-expressing (green) tumors. Data for three representative animals per group are shown. (D) Minimal

residual disease (remaining tumor cells) after MYC inactivation was measured by bioluminescence imaging. (E) Angiogenesis was measured 0 and 4 days after MYC inactivation in control, CD47-expressing, and PD-L1-expressing tumors growing in WT FVB hosts by immunofluorescence for CD31. For representative images, see fig. S15. (F) Control, CD47-expressing, and PD-L1-expressing tumors [as described in (E)] were analyzed by immunostaining for senescence-associated β -gal. The y axis denotes the number of positively-staining microvessels (E) or cells (F) per field. For representative images, see fig. S15B. Data represent mean $\pm$ SEM derived from measurements of three independent tumors and three measurements per tumor. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

and maintenance of MYC-driven tumorigenesis (Fig. 4). The overexpression of MYC may be one general mechanism by which tumor cells up-regulate the expression of immune checkpoint regulators, thereby evading immune surveillance. MYC inactivation has been proposed to restore the immune response against tumors (fig. S17) (39–41).

The suppression of MYC rapidly resulted in decreased mRNA and protein expression of CD47 and PD-L1, which suggests a transcriptional regulatory mechanism. MYC is a general transcriptional amplifier that can regulate gene expression through a multitude of mechanisms (32–34). However, as noted above, MYC also exhibits transcriptional effects dependent on gene dosage (36, 42). The relatively high levels of MYC expression that are associated with rapid proliferation and tumorigenesis may induce CD47 and PD-L1 expression.

Because MYC regulates transcription of their genes, CD47 and PD-L1 may be expressed at higher levels in the steady state as compared with other membrane proteins in tumors. Notably, MYC activation of the *CD47* and *PD-L1* genes appears to require higher levels of MYC binding to the *CD47* and *PD-L1* promoters compared with genes involved in normal cell growth; they may, therefore, represent promoters that have been invaded by oncogenic MYC levels (33, 42). Thus, these genes may be particularly sensitive to MYC withdrawal.

Through the suppression of immune surveillance against tumor cells, MYC activation may influence cancer immunoediting. We propose that during tumor evolution, high levels of MYC expression result in increased expression of CD47 and PD-L1, suppressing both the innate and adaptive immune responses and favoring tumor growth (fig. S17). Upon MYC inactivation, loss of the “don’t find me” and “don’t eat me” signals allows for the destruction of residual tumor cells and, consequently, sustained tumor regression.

Although the effects of MYC on the expression of CD47 and PD-L1 were modest, the consequences on tumor regression were dramatic, consistent with reports that small influences on immune regulators can have marked effects (25). Both CD47 and PD-L1 may also contribute to the tumor microenvironment via their influence on T cell activation and angiogenesis (13, 14, 22, 43–45). CD47 is the receptor for thrombospondin-1, which may regulate cellular programs including angiogenesis, self-renewal, and senescence (13, 14, 45). We speculate that therapies suppressing MYC expression and activity may restore an immune response against human cancers. Humans cancers that overexpress MYC may be especially vulnerable to an immune checkpoint blockade.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S17
Table S1
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METABOLISM

Complementation of mitochondrial electron transport chain by manipulation of the NAD⁺/NADH ratio

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A decline in electron transport chain (ETC) activity is associated with many human diseases. Although diminished mitochondrial adenosine triphosphate production is recognized as a source of pathology, the contribution of the associated reduction in the ratio of the amount of oxidized nicotinamide adenine dinucleotide (NAD⁺) to that of its reduced form (NADH) is less clear. We used a water-forming NADH oxidase from *Lactobacillus brevis* (*LbNOX*) as a genetic tool for inducing a compartment-specific increase of the NAD⁺/NADH ratio in human cells. We used *LbNOX* to demonstrate the dependence of key metabolic fluxes, gluconeogenesis, and signaling on the cytosolic or mitochondrial NAD⁺/NADH ratios. Expression of *LbNOX* in the cytosol or mitochondria ameliorated proliferative and metabolic defects caused by an impaired ETC. The results underscore the role of reductive stress in mitochondrial pathogenesis and demonstrate the utility of targeted *LbNOX* for direct, compartment-specific manipulation of redox state.

A decline in electron transport chain (ETC) activity has been linked to numerous human disorders, ranging from rare genetic syndromes to common diseases, such as neurodegeneration, cancer, and diabetes, as well as the aging process itself (1, 2). How a

decline in ETC activity gives rise to the spectrum of observed pathology cannot be readily explained by a simple deficiency in adenosine triphosphate (ATP) production (1). A key challenge in deciphering mitochondrial pathogenesis stems from the fact that the ETC performs at

least two coupled functions: redox transfer of electrons from NADH [the reduced form of nicotinamide adenine dinucleotide (NAD⁺)] to oxygen and a simultaneous conversion of the free energy of the electromotive force into a proton gradient across the mitochondrial inner membrane. In principle, pathology could stem from an excess of reducing equivalents (termed reductive stress or pseudohypoxia, which includes stalling of NAD⁺-coupled reactions) or a reduced

proton gradient (impairing pH and voltage-coupled processes, including ATP synthesis by the F₁F₀-ATP synthase). Currently, there are no methods for dissecting the redox function of the ETC from its proton pumping function.

Here, we report the application of a genetically encoded tool for compartment-specific manipulation of the NAD⁺/NADH ratio. Our tool is based on the flavin adenine dinucleotide (FAD)-dependent H₂O-forming NADH oxidases, which catalyze the four-electron reduction of O₂ to two molecules of H₂O (Fig. 1A). We focused on bacterial oxidases with specificity for NADH over reduced nicotinamide adenine dinucleotide phosphate (NADPH) (3–7), whose natural function is protection of redox balance and defense against oxygen toxicity (8). Such oxidases have been successfully expressed in bacteria and yeast

for biotechnological applications (9–11). We screened several H₂O-forming NADH oxidases by expressing their human codon-optimized, epitope-tagged versions in cultured human cancer-derived epithelial (HeLa) cells. The enzyme from *Lactobacillus brevis* (LbNOX) was most highly expressed and had the highest oxidase activity when targeted to mitochondria (fig. S1).

We evaluated the biochemical properties of recombinant LbNOX modified to contain a C-terminal FLAG tag and a cleavable N-terminal hexahistidine tag. Purified LbNOX-FLAG has a yellow color in solution and a characteristic ultraviolet (UV)-visible absorption spectrum (major absorption peaks at 371 and 444 nm) consistent with the presence of FAD, which can be reduced upon the addition of sodium dithionite (Fig. 1B). Our recombinant enzyme consumes

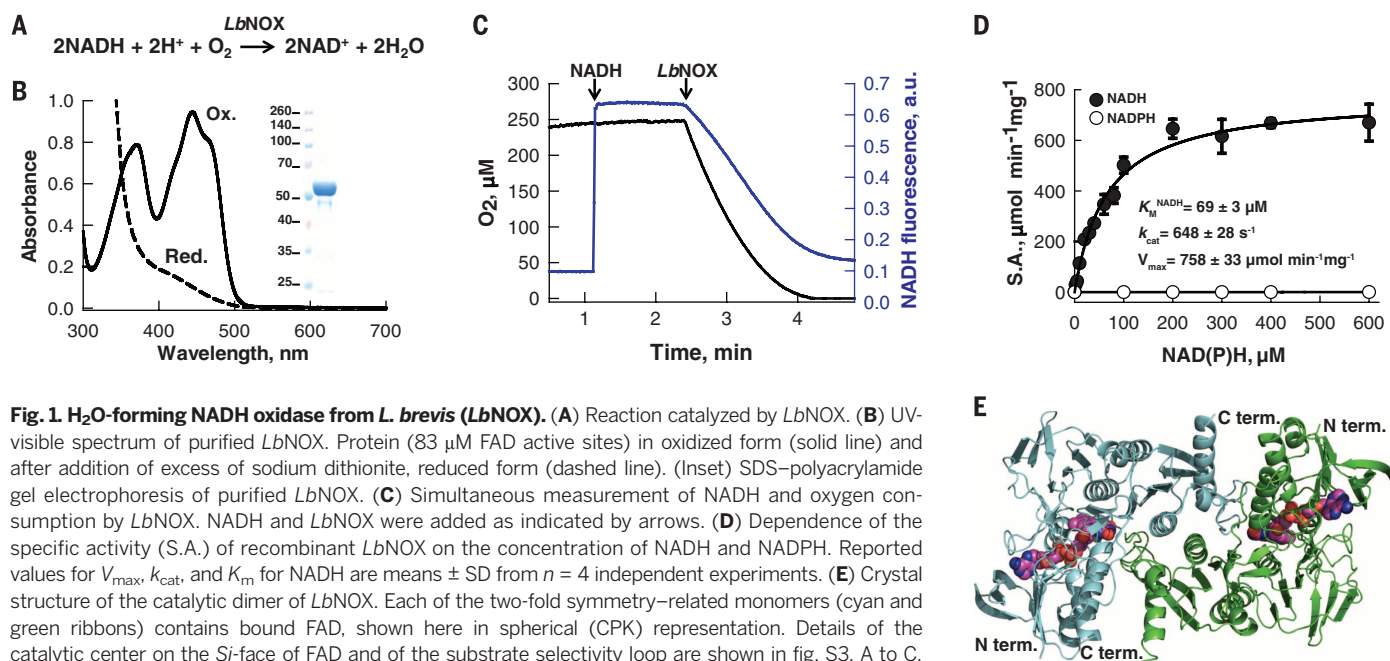


Fig. 1. H₂O-forming NADH oxidase from *L. brevis* (LbNOX). (A) Reaction catalyzed by LbNOX. (B) UV-visible spectrum of purified LbNOX. Protein (83 μM FAD active sites) in oxidized form (solid line) and after addition of excess of sodium dithionite, reduced form (dashed line). (Inset) SDS-polyacrylamide gel electrophoresis of purified LbNOX. (C) Simultaneous measurement of NADH and oxygen consumption by LbNOX. NADH and LbNOX were added as indicated by arrows. (D) Dependence of the specific activity (S.A.) of recombinant LbNOX on the concentration of NADH and NADPH. Reported values for V_{max} , k_{cat} , and K_m for NADH are means $\pm$ SD from $n = 4$ independent experiments. (E) Crystal structure of the catalytic dimer of LbNOX. Each of the two-fold symmetry-related monomers (cyan and green ribbons) contains bound FAD, shown here in spherical (CPK) representation. Details of the catalytic center on the Si-face of FAD and of the substrate selectivity loop are shown in fig. S3, A to C.

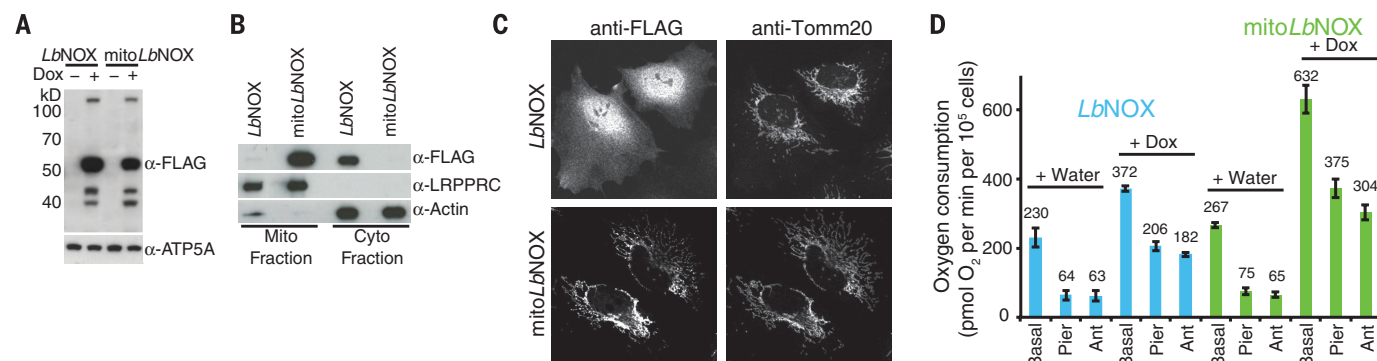


Fig. 2. Expression and activity of LbNOX in human cells. (A) Western blot of LbNOX and mitoLbNOX in HeLa cells after 24-hour induction with water or doxycycline (300 ng/ml) (Dox). Representative gel from one of three independent experiments. (B) Subcellular localization of LbNOX and mitoLbNOX in HeLa cells determined by cell fractionation. LRPPRC is a mitochondrial marker and actin is a cytosolic marker. Representative gel

from one of three independent experiments. (C) Subcellular localization of LbNOX and mitoLbNOX in HeLa cells determined by using fluorescence microscopy. Tomm20 is a marker of mitochondria. (D) Effect of LbNOX and mitoLbNOX expression in HeLa cells on basal, piericidin-resistant, and antimycin-resistant oxygen consumption measured with an XF24 extracellular flux analyzer. Means $\pm$ SEM, $n = 3$ independent experiments.

oxygen and is strictly specific for NADH rather than NADPH with the Michaelis constant (K_m) for NADH of $69 \pm 3 \mu\text{M}$, the maximum velocity (V_{max}) of $758 \pm 33 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, and the turnover number (k_{cat}) of $648 \pm 28 \text{ s}^{-1}$, which is more active than previously reported (3, 12) (Fig. 1, C and D). The molecular size of *LbNOX*-FLAG was determined to be $197 \pm 4 \text{ kD}$, which indicates that the protein is a tetramer in solution. Although enzymes in this family often produce H_2O_2 , *LbNOX*-FLAG produces amounts of H_2O_2 that constitute only 1 to 2% of the amount of H_2O produced during its catalytic cycle (fig. S2A) (4, 6, 7). The apparent K_m for O_2 of *LbNOX*-FLAG was below $2 \mu\text{M}$ ($\sim 0.17\% \text{ O}_2$), as estimated from enzyme-monitored turnover experiments (fig. S2B), which is less than 1/10th of the concentration of oxygen in human venous blood (13). Thus, we expect *LbNOX* to be active in most animal tissues. The enzymatic properties of *LbNOX*-FLAG in solution were well founded in the $2.4 \text{ \AA}$ resolution x-ray structure of this protein that we determined (Fig. 1E, fig. S3, and table S1). Our structure is generally similar to the reported structures of H_2O -forming the reduced form of nicotinamide adenine dinucleotide phosphate NAD(P)H oxidases from *L. sanfranciscensis* (PDB

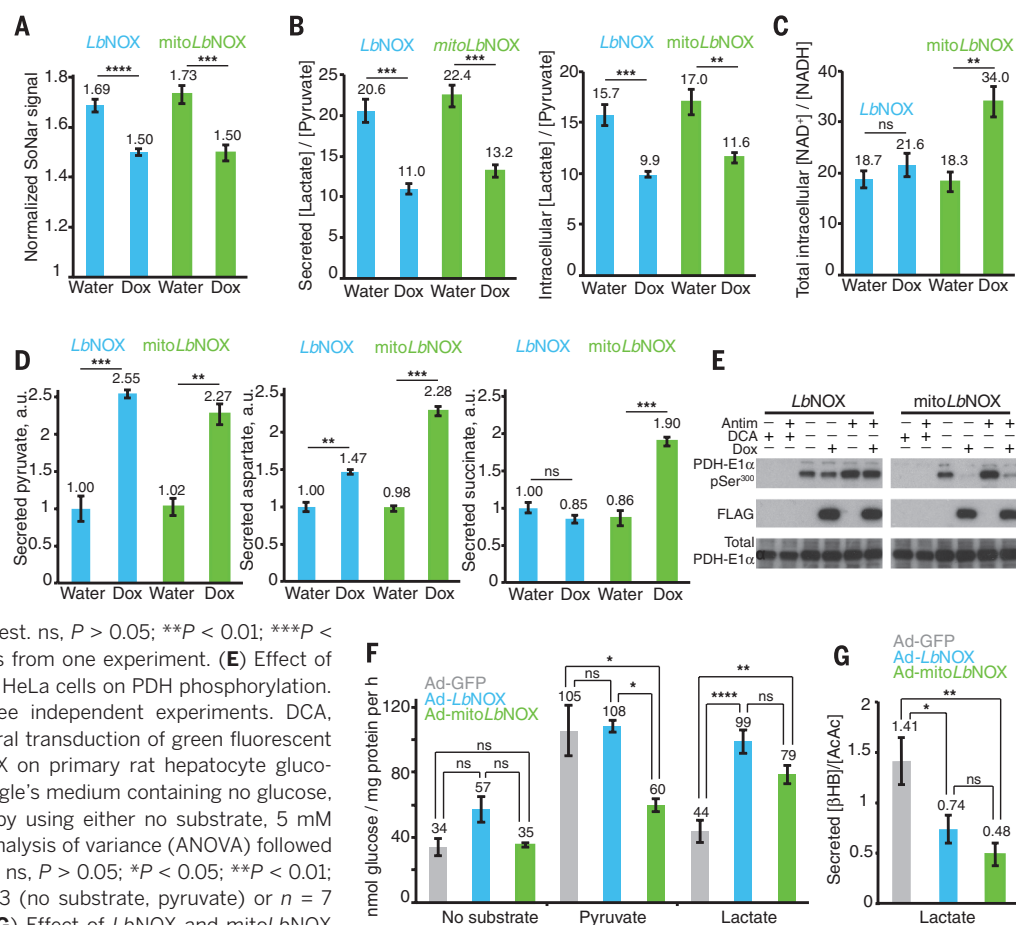
ID 2CDU) and *Streptococcus pyogenes* (PDB ID 2BCO) (14, 15). However, our structure captures *LbNOX* in a new state with molecular oxygen (O_2) bound and the redox active Cys⁴² in a reduced form (Cys⁴²-SH) (fig. S3). [See supplementary materials (SM) for a detailed discussion of the x-ray structure.] In conclusion, the high selectivity for NADH over NADPH, negligible H_2O_2 production, and very low K_m for O_2 made *LbNOX* attractive for additional studies in human cells.

To determine whether we could express *LbNOX*-FLAG safely and efficaciously in various compartments of human cells, we used lentiviral infection to generate HeLa cells that expressed untargeted or mitochondria-targeted human codon-optimized *LbNOX*-FLAG (referred to as *LbNOX* and *mitoLbNOX* henceforth) under the control of a doxycycline-inducible promoter (TRE3G) (Fig. 2A and fig. S1A). We used fluorescence microscopy and cell fractionation to confirm diffuse localization of *LbNOX* and mitochondrial localization of *mitoLbNOX* (Fig. 2, B and C). Cells appeared grossly normal without any impact on cell proliferation or production of reactive oxygen species (ROS) (fig. S4, A and B). As expected, expression of *LbNOX* and *mitoLbNOX* in HeLa cells increased oxygen consumption by

1.6- and 2.4-fold, respectively (Fig. 2D and fig. S4C). The increase was resistant to ETC inhibitors, which indicates that it resulted from *LbNOX* oxidase activity and not from the increased ETC activity. Despite similar expression levels (Fig. 2A), *mitoLbNOX* induced a larger increase in oxygen consumption than *LbNOX* (Fig. 2D), likely because of the higher concentration of NADH within mitochondria (16–18). It is important to remember that in converting NADH to NAD^+ , *LbNOX* also consumes protons and oxygen and, therefore, could affect cellular pH or oxygen levels, depending on experimental context.

We determined the impact of expressing *LbNOX* or *mitoLbNOX* on cellular concentrations of NAD^+ and NADH (Fig. 3 and fig. S5). We used a genetic sensor, SoNar (19), to measure cytosolic NADH. SoNar is a fusion of circularly permuted yellow fluorescent protein and a modified NADH-binding protein, Rex, from *Thermus aquaticus*. Binding of NADH to SoNar leads to an increase in fluorescence. Expression of *LbNOX* or *mitoLbNOX* decreased the fluorescence signal from SoNar, which indicated that both *LbNOX* and *mitoLbNOX* decrease cytosolic NADH (Fig. 3A and fig. S5B). Consistent with this result, intracellular and secreted lactate/pyruvate ratios, traditionally used

Fig. 3. Effect of *LbNOX* and *mitoLbNOX* on NAD^+/NADH ratios, metabolic fluxes, PDH phosphorylation, and gluconeogenesis. (A to C) Effect of *LbNOX* and *mitoLbNOX* expression in HeLa cells on (A) cytoplasmic NADH concentrations determined with fluorescence microscopy using SoNar-expressing cells ($n = 7$), (B) intracellular and secreted lactate/pyruvate ratio determined by liquid chromatography–mass spectrometry (LC-MS) ($n = 4$), and (C) intracellular NAD^+/NADH ratios determined by HPLC ($n = 4$). Student's t test. ns, $P > 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$. Means $\pm$ SEM. (D) Effect of *LbNOX* and *mitoLbNOX* expression in HeLa cells on release rate of pyruvate, aspartate, and succinate, determined by comparing concentrations in spent versus fresh media. Student's t test. ns, $P > 0.05$; $**P < 0.01$; $***P < 0.001$. Means $\pm$ SEM, $n = 3$ replicates from one experiment. (E) Effect of *LbNOX* and *mitoLbNOX* expression in HeLa cells on PDH phosphorylation. Representative gel from one of three independent experiments. DCA, dichloroacetate. (F) Effect of adenoviral transduction of green fluorescent protein (GFP), *LbNOX*, or *mitoLbNOX* on primary rat hepatocyte gluconeogenesis in Dulbecco's modified Eagle's medium containing no glucose, no glutamine, and no pyruvate and by using either no substrate, 5 mM pyruvate, or 5 mM lactate. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. ns, $P > 0.05$; $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$. Means $\pm$ SEM, $n = 3$ (no substrate, pyruvate) or $n = 7$ (lactate) independent experiments. (G) Effect of *LbNOX* and *mitoLbNOX* on the secreted β -hydroxybutyrate/acetoacetate ratio in rat hepatocytes performing gluconeogenesis from lactate as a substrate. Metabolite levels determined by using LC-MS. One-way ANOVA followed by Tukey's multiple comparisons test. ns, $P > 0.05$; $*P < 0.05$; $**P < 0.01$. Means $\pm$ SEM, $n = 10$ independent experiments.



as proxies for the cytosolic NADH/NAD⁺ ratio (17), decreased in cells expressing *LbNOX* or *mitoLbNOX* (Fig. 3B and fig. S5D). The ratio of total cellular NAD⁺ to total NADH, based on high-performance liquid chromatography (HPLC) measurements, was increased twofold by *mitoLbNOX*, whereas *LbNOX* did not have a significant effect (Fig. 3C and fig. S5C). Perturbation of the total NAD⁺/NADH ratio likely reflects changes in amounts of mitochondrial NADH, because most of the effect on the ratio resulted from changes in NADH concentration (fig. S5A), and most of the NADH inside the cell is present in mitochondria. The latter effect is supported by fractionation experiments (20) and by the observation that the majority of NAD(P)H autofluorescence in cells comes from mitochondrial NADH (21). In summary, *LbNOX* and *mitoLbNOX* can be used to perturb the NAD⁺/NADH ratio, and our compartment-specific measurements of HeLa cells (Fig. 3, A to C) indicate that, although perturbation of the mitochondrial NAD⁺/NADH ratio leads to changes in the cytosolic NAD⁺/NADH ratio, the converse is not true.

We performed metabolic profiling on medium in which cells expressing *LbNOX* or *mitoLbNOX* had been grown (Fig. 3D and fig. S6, A and B). We identified pyruvate, aspartate, and succinate as three metabolites whose consumption or release was changed more than twofold (Student's *t* test; *P* < 0.01) by either enzyme. These changes are attributable to compartment-specific changes of NAD⁺/NADH by *LbNOX* or *mitoLbNOX* (see SM for discussion). It is notable that *LbNOX* and *mitoLbNOX* did not have a significant effect on the uptake of glucose and release of lactate (fig. S6C).

In vitro phosphorylation of mitochondrial pyruvate dehydrogenase (PDH) is regulated by the NAD⁺/NADH ratio (22), but this has not been shown directly in intact cells. Treatment of HeLa cells with dichloroacetate, an inhibitor of pyruvate dehydrogenase kinases (PDKs), inhibits phosphorylation of PDH, and antimycin treatment, which blocks the ETC and decreases the mitochondrial NAD⁺/NADH ratio, increases PDH phosphorylation. In agreement with in vitro studies, PDH was almost completely dephosphorylated in the presence of *mitoLbNOX* but not *LbNOX* (Fig. 3E and fig. S6D). The data on PDH phosphorylation are consistent with our observation that *mitoLbNOX*, but not *LbNOX*, increases the mitochondrial NAD⁺/NADH ratio in HeLa cells (Fig. 3C).

We expressed *LbNOX* and *mitoLbNOX* in primary rat hepatocytes to study gluconeogenesis. The cytosolic NAD⁺/NADH ratio has been reported to affect gluconeogenesis, although classical approaches relied on indirect methods for manipulating the redox state (23, 24). In our hepatocyte system, rates of gluconeogenesis were significantly higher if pyruvate rather than lactate was used as a substrate, which we attribute to the NAD⁺/NADH ratio-dependent inhibition of lactate-to-pyruvate conversion (23). Consistent with this hypothesis, rates of gluconeogenesis from lactate were increased to those seen with

pyruvate when primary hepatocytes expressed *LbNOX*, whereas gluconeogenesis from pyruvate was not affected (Fig. 3F). Gluconeogenesis from lactate was also increased by *mitoLbNOX*. Gluconeogenesis from pyruvate, however, was inhibited by *mitoLbNOX*, perhaps because strong oxidation of mitochondrial NADH prevents formation of malate (25). In assays of gluconeogenesis using lactate as a precursor, expression of either *LbNOX* or *mitoLbNOX* decreased the ratio of secreted β -hydroxybutyrate/acetoacetate (Fig. 3G), the classical proxy for the mitochondrial NADH/NAD⁺ ratio (17). Although the NAD⁺/NADH ratio appears to be increased, we cannot exclude the possibility that *LbNOX* or *mitoLbNOX* induce hypoxia. *LbNOX* evidently increased the mitochondrial NAD⁺/NADH ratio in rat hepatocytes (Fig. 3G) but not in HeLa cells (Fig. 3C), which might reflect differences in cell type or media conditions.

Mammalian cells lacking a functional ETC require the addition of exogenous pyruvate and uridine for cell proliferation (26–28). Uridine is required because one of the enzymes in de novo uridine biosynthesis (dihydroorotate dehydrogenase) is coupled to the ETC through coenzyme Q. The requirement for pyruvate, however, has been less clear because it participates in many reactions but has been proposed to rescue cell growth by recycling NAD⁺ from NADH through cytosolic lactate dehydrogenase (26, 29). If the NAD⁺ recycling hypothesis is correct, then supplementation with oxaloacetate should have the same effect as pyruvate, because it can be reduced by malate dehydrogenase while recycling NAD⁺. Oxaloacetate, like pyruvate, rescued the proliferation defect induced by piericidin, whereas malate and lactate did not (Fig. 4A). Alpha-ketobutyrate also rescues the proliferative defect induced by ETC inhibition (30). Furthermore, a large number of α -keto acids can rescue the pyruvate dependence of proliferation in cells with intact ETC (31). These findings support the NAD⁺-recycling hypothesis, although they are still indirect, as α -keto acids have many metabolic roles.

We used *LbNOX* to directly test whether NAD⁺ recycling is an essential function of the ETC that is required for cell proliferation. We inhibited ETC function, with piericidin (a complex I inhibitor), antimycin (a complex III inhibitor), ethidium bromide (a mitochondrial DNA replication inhibitor), and chloramphenicol (an inhibitor of mitochondrial translation) in HeLa cells supplemented with uridine but lacking pyruvate. HeLa cells cannot proliferate in these conditions (Fig. 4B and fig. S7). Expression of either *LbNOX* or *mitoLbNOX* rescued cell proliferation in the presence of these ETC inhibitors, which indicated that regeneration of NAD⁺ in either cytosol or mitochondria is sufficient to complement ETC activity that is required for cell proliferation (Fig. 4B). Metabolic profiling showed that of the nine metabolites whose uptake or release is affected greater than twofold by antimycin (Student's *t* test; *P* < 0.01), all could be reversed by either *LbNOX* or *mitoLbNOX*,

which reflects a metabolic rescue (fig. S8) (see SM for discussion). Our metabolic profiling data are complementary to recent studies demonstrating an inhibition of aspartate biosynthesis in cells with dysfunctional ETC (30, 32, 33). As a control, we also showed that the rescue by *LbNOX* or *mitoLbNOX* was not attributable to an effect on mitochondrial membrane potential (fig. S9, A to C), nor was it due to a rescue of ETC-derived ATP synthesis (fig. S9, D to G).

Collectively, these studies (Fig. 4 and figs. S7 to S9) underscore the importance of NAD⁺ recycling by the ETC to support proliferation. In healthy cells, the ETC produces ATP and simultaneously recycles mitochondrial NADH to NAD⁺, with a secondary oxidation of cytosolic NADH via shuttles. In the absence of a functional ETC, glycolysis is capable of compensating for the lack of ATP production, but it is net redox-neutral.

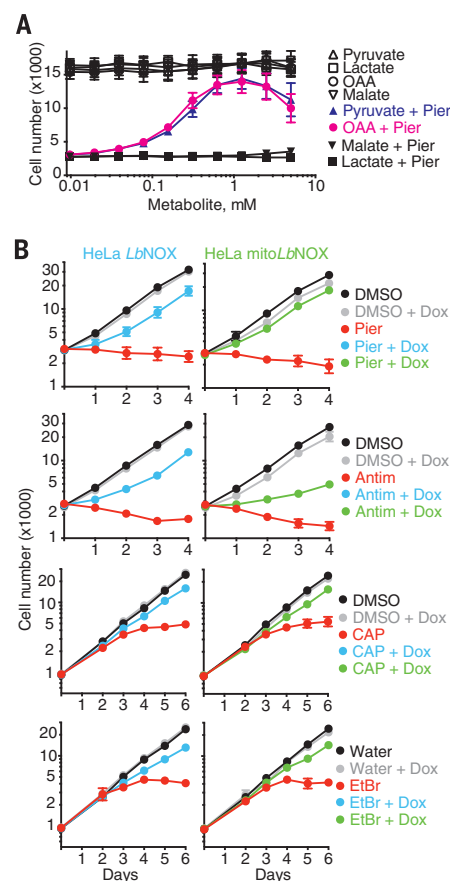


Fig. 4. NAD⁺ recycling rescues proliferation in cells with impaired ETC. (A) Effect of pyruvate, oxaloacetate, lactate, and malate addition on proliferation of HeLa Tet3G luciferase cells in the presence of 200 μ M uridine and in the presence or absence of 1 μ M piericidin. Means $\pm$ SEM, *n* = 5 independent experiments. (B) Effect of *LbNOX* and *mitoLbNOX* expression in HeLa cells on inhibition of cell proliferation by 1 μ M piericidin, 1 μ M antimycin, 10 μ g/ml chloramphenicol, and 30 ng/ml ethidium bromide (EtBr) in the presence of 200 μ M uridine. DMSO, dimethyl sulfoxide. Means $\pm$ SEM, *n* = 3 independent experiments.

NAD⁺ recycling is likely key for cell proliferation, because many biosynthetic pathways produce NADH as a byproduct (34). These insights confirm the long-standing hypothesis (26, 29) that pyruvate supplementation rescues proliferation in cells with disrupted ETC by restoring NAD⁺/NADH balance via the LDH reaction.

In the future, *LbNOX* and engineered or naturally occurring variants may become valuable tools for studying compartmentalization of redox metabolism. These constructs will allow for a dissection of the relative contributions of redox imbalance and ATP insufficiency to mitochondrial disease pathogenesis. If a substantial amount of the organ pathology of mitochondrial disease stems from reductive stress or pseudohypoxia, then expression of this single polypeptide holds promise as a “protein prosthesis” for the large number of disorders characterized by ETC dysfunction.

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Hospital has filed a patent application on the technology described in this paper. Atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession number 5ERO.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/352/6282/231/suppl/DC1
Materials and Methods
Figs. S1 to S9
Tables S1 to S3
References (35–52)

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HUMAN GENOMICS

Excavating Neandertal and Denisovan DNA from the genomes of Melanesian individuals

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Although Neandertal sequences that persist in the genomes of modern humans have been identified in Eurasians, comparable studies in people whose ancestors hybridized with both Neandertals and Denisovans are lacking. We developed an approach to identify DNA inherited from multiple archaic hominin ancestors and applied it to whole-genome sequences from 1523 geographically diverse individuals, including 35 previously unknown Island Melanesian genomes. In aggregate, we recovered 1.34 gigabases and 303 megabases of the Neandertal and Denisovan genome, respectively. We use these maps of archaic sequences to show that Neandertal admixture occurred multiple times in different non-African populations, characterize genomic regions that are significantly depleted of archaic sequences, and identify signatures of adaptive introgression.

For much of human history, modern humans overlapped in time and space with other hominins (1). Analyses of the Neandertal (2, 3) and Denisovan (4, 5) genomes revealed that gene flow occurred between these archaic hominins and the ancestors of modern humans (3–8). Consequently, all non-African populations derive ~2% of their ancestry from Neandertals (3), whereas substantial levels of Denisovan ancestry (~2 to 4%) are only found in Oceanic populations (5), although low levels of Denisovan ancestry may be more widespread (9, 10). Recently, catalogs of introgressed Neandertal sequences have been

created in Eurasians (11, 12). However, considerably less is known about the genomic organization and characteristics of Denisovan sequences that persist in modern individuals.

We sequenced 35 individuals from 11 locations in the Bismarck Archipelago of Northern Island Melanesia, Papua New Guinea (Fig. 1A) (13, 14), to a median depth of 40x (tables S1 and S2), including a trio to facilitate haplotype reconstruction. Unless otherwise noted, analyses were performed on a subset of 27 unrelated individuals (14). We integrated our sequencing data with single-nucleotide polymorphism genotypes from 1937 individuals spanning 159 worldwide populations (Fig. 1A and table S3) (14–16). A global principal components analysis (PCA) and ADMIXTURE analysis shows that our samples cluster most closely with other Oceanic individuals (Fig. 1B and fig. S1), and population sizes inferred by pairwise sequentially Markovian coalescent analysis are consistent with other non-African populations (fig. S2) (14). Furthermore, our Melanesian samples show genetic similarities to both Neandertals and Denisovans, whereas all other non-African populations only exhibit affinity toward Neandertals (Fig. 1C and fig. S3) (14). Using an *f₄* statistic (14, 15, 17), we find significant evidence of Denisovan

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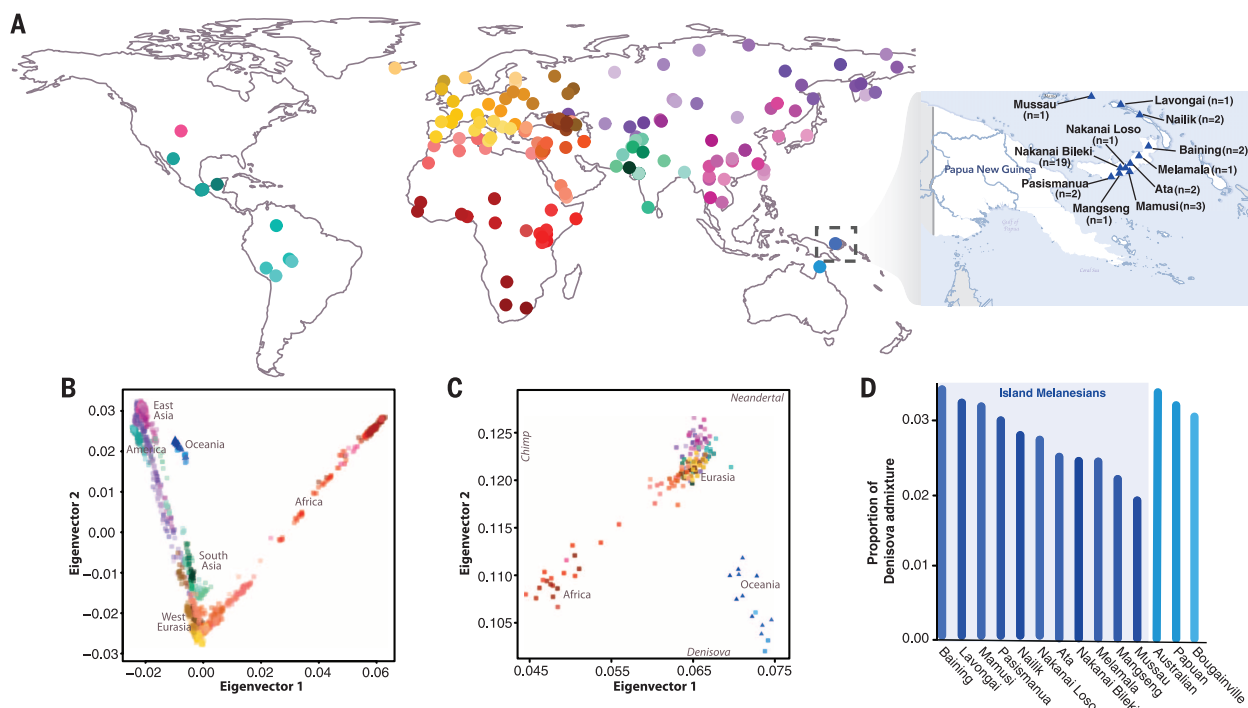


Fig. 1. Melanesian genomic variation in a global context. (A) Locations of the 159 geographically diverse populations studied. Information about the Melanesian individuals sequenced (blue triangles) is shown in the inset. (B) PCA of Melanesian genomes in the context of present-day worldwide genetic diversity. (C) Modern human variation projected onto the top two eigenvectors defined by PCA of the Altai Neandertal, Denisovan, and chimpanzee genome (14). Population means were plotted for each of the 11 Melanesian populations and each population of the global data set. (D) Estimates of Denisovan ancestry in Oceanic populations estimated from an f_4 statistic (14). The 11 Melanesian populations are highlighted by the light blue box.

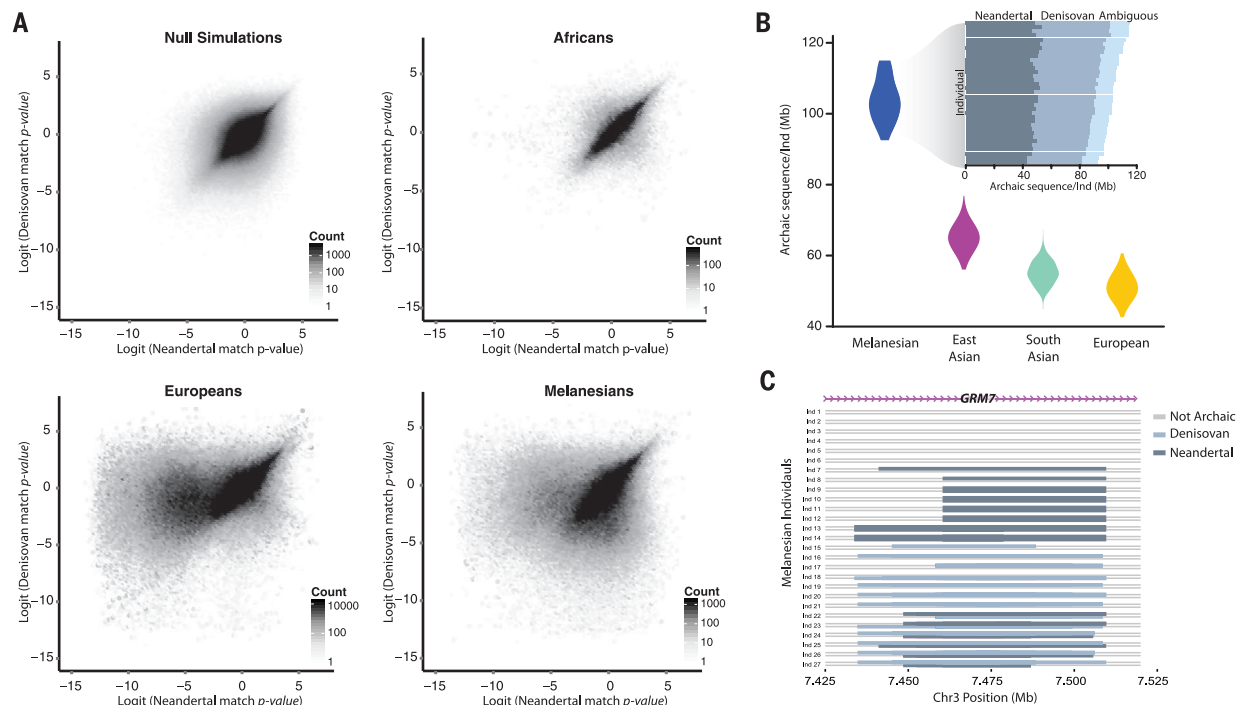


Fig. 2. Identifying Neandertal and Denisovan sequences in modern human genomes. (A) Bivariate archaic match P value distributions for simulations of nonintrogressed sequences, Esan in Nigeria, Europeans, and Melanesians. Null simulations and Esan show no skew in Neandertal or Denisovan match P values toward zero, Europeans show only a skew of Neandertal match P values toward zero, and Melanesians exhibit both Neandertal and Denisovan match P values

skewed toward zero. (B) Amount of archaic introgressed sequences identified in each population. (Inset) Amount of Neandertal, Denisovan, and ambiguous (Neandertal or Denisovan) introgressed sequences for each Melanesian individual. (C) Schematic representation of introgressed haplotypes in an intronic portion of the *GRM7* locus in Melanesian individuals illustrating mosaic patterns of archaic ancestry.

ancestry ($Z > 4$) in our Melanesian samples, with admixture proportions varying between 1.9 and 3.4% (Fig. 1D and table S4).

Having demonstrated that our Melanesian individuals have both Neandertal and Denisovan ancestry, we developed an approach to recover and classify archaic sequences. Briefly, we first identify putative introgressed sequences using the statistic S^* (which does not use information from an archaic reference genome) (11, 18) and then refine this set by comparing significant S^* haplotypes to the Neandertal and Denisovan genomes and testing to determine whether they match more than expected by chance. Variation in neutral divergence between archaic groups across loci and incomplete lineage sorting complicate classification of archaic haplotypes as Neandertal or Denisovan (fig. S4)

(14). To address this issue, we developed a likelihood method that operates on the bivariate distribution of Neandertal and Denisovan match P values (14). This framework estimates the proportion of Neandertal, Denisovan, and null sequences in the set of S^* significant haplotypes, identifies archaic haplotypes at a desired false discovery rate (FDR), and probabilistically categorizes them as Neandertal, Denisovan, or ambiguous (i.e., Neandertal or Denisovan status cannot be confidently distinguished) (14). In addition to our Melanesian samples, we also applied our method to whole-genome sequences from 1496 geographically diverse individuals studied as part of the 1000 Genomes Project (table S5) (14, 19).

We evaluated our approach through coalescent simulations (figs. S5 and S6) and by analyzing Af-

rican populations (fig. S7). Archaic match P values calculated from null coalescent simulations without archaic admixture and significant S^* sequences in African individuals show similar distributions (Fig. 2A), consistent with little to no Neandertal or Denisovan ancestry in most African populations. Notably, Luhya and Gambians do show evidence of having some Neanderthal ancestry (fig. S8 and table S6), most likely inherited indirectly through recent admixture with non-Africans (20). In Europeans, we see a strong skew of Neandertal, but not Denisovan, match P values toward zero (Fig. 2A). In contrast, Melanesians exhibit a marked skew of both Neandertal and Denisovan match P values toward zero (Fig. 2A).

In aggregate, across all 1523 non-African individuals analyzed, we recovered 1340 Mb and 304 Mb

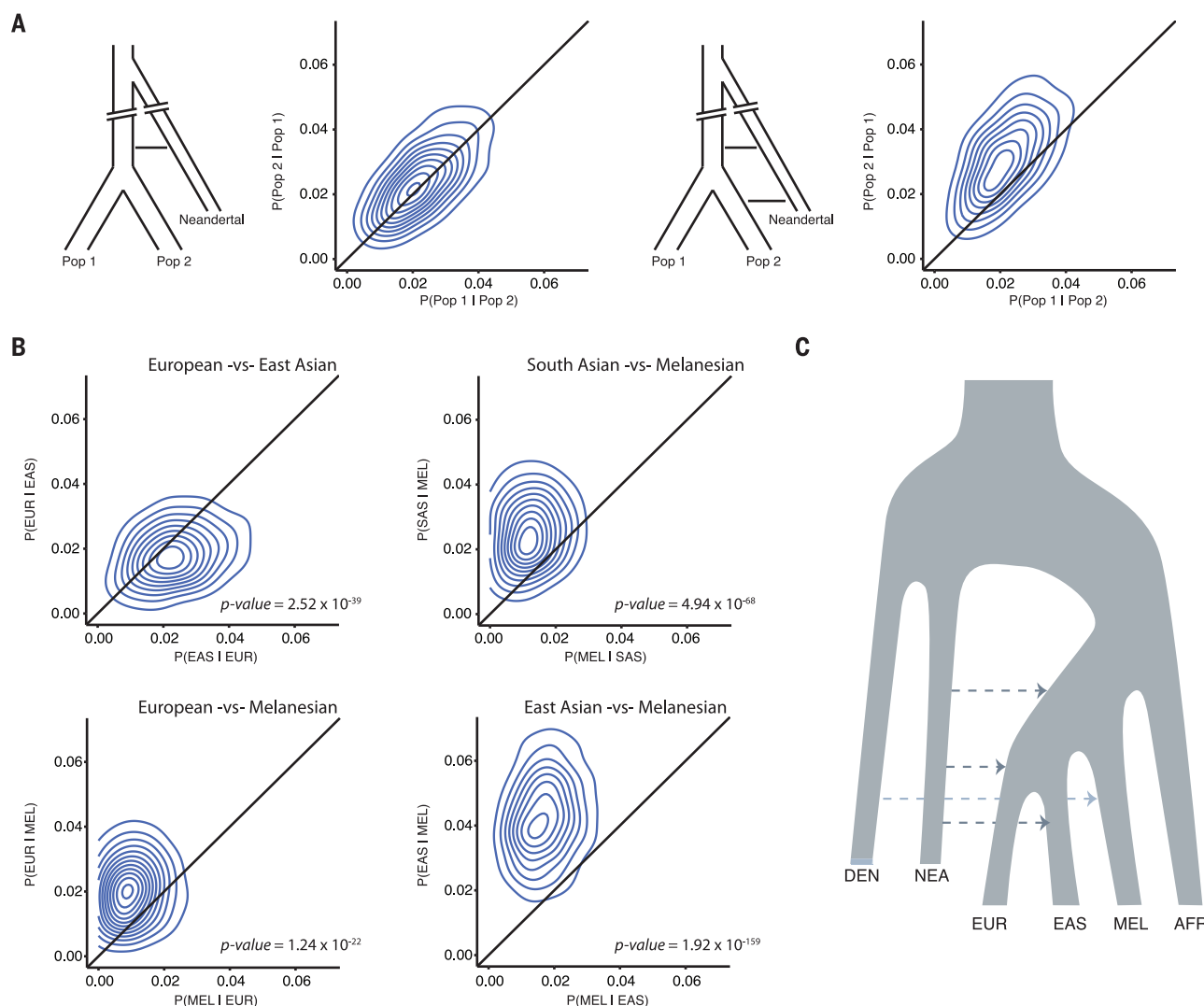


Fig. 3. Identifying shared and unique pulses of Neandertal admixture among human populations. (A) Schematics of two simulated introgression models and patterns of reciprocal match probabilities. Contour plots are fit to the scatter plot of reciprocal match probabilities calculated from analyzing all pairwise combinations of individuals between two populations. (Left) Gene flow occurs into the common ancestor of Population 1 and Population 2, and reciprocal match probabilities fall along the diagonal as predicted by theory

(binomial test, $P > 0.05$) (14). Right, Population 2 receives additional admixture shifting reciprocal match probabilities above the diagonal (binomial test, $P < 0.05$). (B) Reciprocal match probabilities of Neandertal sequences in modern human populations, consistent with additional Neandertal admixture into East Asians versus Europeans, and into Europeans, East Asians, and South Asians versus Melanesians. (C) Simplified schematic of admixture history consistent with the data.

of the Neandertal and Denisovan genomes, respectively, at a FDR = 5%. Melanesian individuals have on average 104 Mb of archaic sequences per individual (48.9, 42.9, and 12.2 Mb of Neandertal, Denisovan, and ambiguous sequence, respectively) (Fig. 2B). In contrast, we only call between 0.026 Mb (in Esan) to 0.5 Mb (in Luhya) of sequences per individual as archaic in Africans, highlighting that our method and error rates are well calibrated. The higher levels of archaic ancestry in Melanesians result in an average of 20 compound homozygous archaic loci per individual, with one Neandertal and one Denisovan haplotype (Fig. 2C and fig. S9). We estimate that 61% of the variability in the amount of Denisovan se-

quences between individuals is explained by variation in Papuan ancestry ($P = 7.8 \times 10^{-4}$) (table S7) (14). In other non-Africans, we identify on average 65.0, 55.2, and 51.2 Mb of archaic sequences in East Asians, South Asians, and Europeans, respectively (Fig. 2B). Virtually all of the archaic sequences in these populations are Neandertal in origin, although a small fraction (<1%) of introgressed sequences in East and South Asians are predicted to be Denisovan (fig. S8 and table S6) (14).

We developed a method to determine whether two populations have shared or unique admixture histories based on patterns of reciprocal sharing of Neandertal sequences among individuals (fig. S10) (14). We validated the expected behavior of

our method in simulated data (Fig. 3A) and confirm previous observations of an additional admixture event unique to East Asians (11, 21, 22) (Fig. 3B). We find evidence for an additional pulse of Neandertal admixture in Europeans, East Asians, and South Asians compared with Melanesians (Fig. 3B), which is robust to different statistical thresholds used to call Neandertal and Denisovan sequences and determining significance (figs. S11 to S13) (14). Conversely, we find no evidence of differences in admixture histories between Europeans and South Asians (fig. S14). Collectively, these data suggest that Neandertal admixture occurred at least three distinct times in modern human history (Fig. 3C). Although most

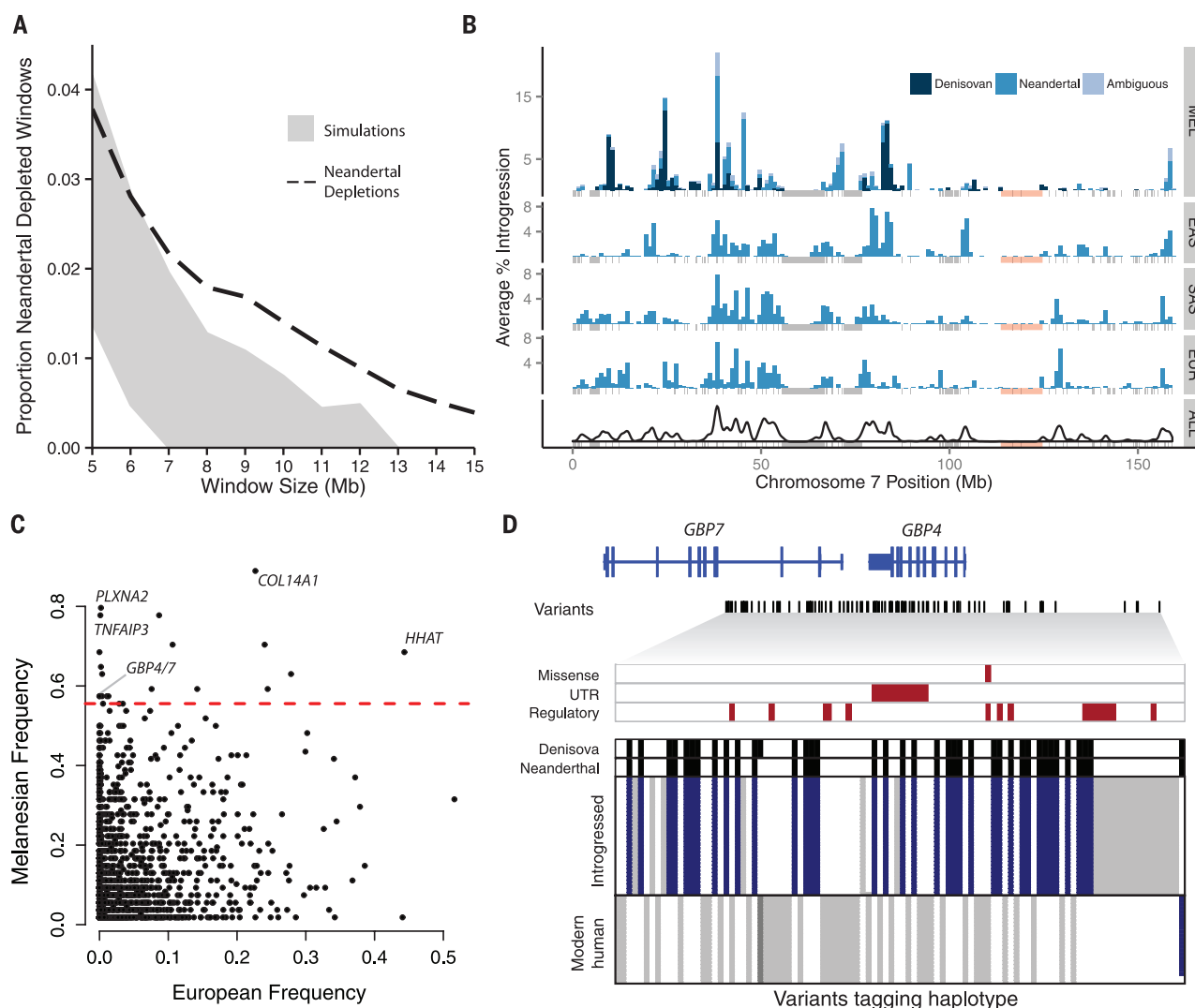


Fig. 4. Maps of archaic admixture reveal signatures of purifying and positive selection. (A) Proportion of windows significantly depleted of Neandertal introgression in Europeans and East Asians (dashed line) versus what is expected in neutral demographic models (95% confidence interval in gray). (B) Distribution of Neandertal and Denisovan sequences across chromosome 7 in Melanesians (MEL), East Asians (EAS), South Asians (SAS), and Europeans (EUR), and then summed across all populations (ALL). Masked regions are shown as gray vertical lines. An 11.1-Mb region significantly depleted of Denisovan and Neandertal ancestry in all populations is shown in light pink. (C) The frequency of archaic haplotypes in Melanesians versus Europeans. The

red line indicates the 99th percentile defined by neutral coalescent simulations. Notable genes are labeled. (D) Visual representation of a high-frequency haplotype encompassing *GBP4* and *GBP7*. Rows indicate individual haplotypes, and columns denote variants that tag the introgressed haplotype (14). Alleles are colored according to whether they are ancestral (white), derived variants that match both archaic genomes (blue), derived variants that match one archaic genome (dark gray), or derived but do not match either archaic genome (light gray). Archaic sequences are represented above, with black denoting derived variants. Missense, untranslated region (UTR), and putative regulatory variants (14) are highlighted with red boxes.

South Asian populations show shared histories of archaic admixture, we find significant evidence of differential Neandertal admixture between some European and East Asian populations (figs. S15 to S17).

The density of surviving Neandertal sequences across the genome is heterogeneous (11), and regions that are strongly depleted of Neandertal ancestry may represent loci where archaic sequences were deleterious in hybrid individuals and were purged from the population. To quantify how unusual Neandertal depleted regions are under neutral models, we performed coalescent simulations (14), focusing on individuals of European and East Asian ancestry whose demographic histories are known in most detail. Depletions of Neandertal sequences that extend ≥ 8 Mb are significantly enriched in the observed compared with simulated data (permutation $P < 0.01$) (Fig. 4A and fig. S18). Neandertal depleted regions that span at least 8 Mb are also significantly (Kolmogorov-Smirnov test, $P < 10^{-15}$) depleted of Neandertal sequences in South Asians and Melanesians (fig. S19 and table S8).

We find significantly more overlap in regions depleted of Neandertal and Denisovan lineages than expected by chance (permutation $P = 0.0008$) (fig. S20 and table S9) (14), consistent with recurrent selection against deleterious archaic sequences. Indeed, deserts of archaic sequences tend to exhibit higher levels of background selection (figs. S21 and S22). Regions depleted of archaic lineages are significantly enriched for genes expressed in specific brain regions, particularly in the developing cortex and adult striatum (permutation $P < 0.05$) (table S10). A large region depleted of archaic sequences spans 11 Mb on chromosome 7 and contains the *FOXP2* gene (Fig. 4B), which has been associated with speech and language (23). This region is also significantly enriched for genes associated with autism spectrum disorders (Fisher's exact test, $P = 0.008$) (14). Although our data show that large regions depleted of archaic ancestry are inconsistent with neutral evolution, mechanisms other than selection, such as structural variation, could also contribute to the appearance of archaic deserts, and thus additional work is necessary to fully understand the origins of such regions.

We identified putative adaptively introgressed sequences in Melanesians by identifying archaic haplotypes at unusually high frequencies, as determined by coalescent simulations under a wide variety of neutral demographic models (14). At a frequency threshold of 0.56, corresponding to the 99th percentile of simulated data, we identified 21 independent candidate regions for adaptive introgression (Fig. 4C and table S12). Fourteen are of Neanderthal origin, three are Denisovan, three are ambiguous, and one segregates both Neanderthal and Denisovan haplotypes. Six regions do not contain any protein-coding genes, and seven high-frequency archaic haplotypes span only a single gene (table S12). High-frequency archaic haplotypes overlap several metabolism-related genes, such as *GCG* (a hormone that increases blood glucose levels) and *PLPPI* (a membrane protein involved in lipid metabolism). Moreover, five regions

either span or are adjacent to immune-related genes, including a haplotype encompassing *GBP4* and *GBP7* (Fig. 4D), which are induced by interferon as part of the innate immune response.

Substantial amounts of Neandertal and Denisovan DNA can now be robustly identified in the genomes of present-day Melanesians, allowing new insights into human evolutionary history. As genome-scale data from worldwide populations continue to accumulate, a nearly complete catalog of surviving archaic lineages may soon be within reach. Key challenges remain, including evaluating the functional and phenotypic consequences of introgressed sequences and refining estimates on the timing, location, and other characteristics of admixture events. Ultimately, maps of surviving Neandertal, Denisovan, and potentially other hominin (1) sequences will help us to interpret patterns of human genomic variation and understand how archaic admixture influenced the trajectory of human evolution.

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SUPPLEMENTARY MATERIALS

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BIOPHYSICS

Direct observation of transition paths during the folding of proteins and nucleic acids

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Transition paths, the fleeting trajectories through the transition states that dominate the dynamics of biomolecular folding reactions, encapsulate the critical information about how structure forms. Owing to their brief duration, however, they have not previously been observed directly. We measured transition paths for both nucleic acid and protein folding, using optical tweezers to observe the microscopic diffusive motion of single molecules traversing energy barriers. The average transit times and the shapes of the transit-time distributions agreed well with theoretical expectations for motion over the one-dimensional energy landscapes reconstructed for the same molecules, validating the physical theory of folding reactions. These measurements provide a first look at the critical microscopic events that occur during folding, opening exciting new avenues for investigating folding phenomena.

Biomolecular folding is famously complex, involving a diffusive search over a multi-dimensional conformational energy landscape for the lowest-energy structure (1). The most critical parts of the folding path-

way, dominating the dynamics, are the transition states, the unstable intermediates through which a molecule must pass when changing conformation (2). A key goal in folding studies has been to observe molecules as they traverse a particular

path through the transition states, providing a direct view of the behavior in the transition states. Such transition paths (Fig. 1A) are very short-lived, however, and are moreover inherently a single-molecule phenomenon, features that make them very challenging to observe experimentally. As a result, although transition paths have been studied in computational simulations (3, 4), folding experiments have primarily used indirect means of characterizing transition states (2). The inability to observe transition paths has also limited direct experimental tests at the microscopic level of the fundamentally diffusive nature of folding.

Recently, advances in single-molecule methods have enabled the first glimpses of the transition paths in folding reactions. By analyzing photon statistics from high-time-resolution single-molecule fluorescence spectroscopy measurements, the average transit time across transition paths, τ_{tp} , was found for two small proteins, α_3D and a WW domain (5, 6), and upper bounds were determined for the protein GB1 (5, 7) and a DNA hairpin (8). Average transition path times were also found indirectly for both protein and nucleic acid folding from force spectroscopy measurements, in which single molecules were unfolded and refolded under tension applied by optical tweezers (9), using diffusive theories to determine the value for τ_{tp} expected from the measured energy landscape for folding (10–12). Statistical features of the transition paths, such as the conditional probability of being on a transition path as a function of the reaction coordinate (13), were also investigated, revealing that the folding was well described by one-dimensional (1D) diffusion (14). Despite these advances, however, it has not been possible to discern directly the properties of individual transition paths.

Here we describe measurements observing transition paths directly in the folding of single molecules, using high-resolution force spectroscopy. Force spectroscopy is an especially powerful tool for studying transition paths, because many transitions can be measured from a single molecule, yielding robust statistics. We first studied the two-state DNA hairpin 30R50/T4 (Fig. 1B, inset), which has been characterized extensively in previous work (10, 14, 15), especially through measurements of the energy landscape underlying its folding properties (16–19). Folding and unfolding transitions of single hairpins connected via DNA handles to beads held in two stiff optical traps (Fig. 1B) were measured in equilibrium at constant trap separation, at a load close to the force required to populate the folded and unfolded states equally, $F_{1/2}$ (20). The time resolution of the measurement was improved more than fivefold from previous studies of τ_{tp} (10, 17), to about 6 to 11 μ s (fig. S1).

From equilibrium trajectories of the extension of the molecule (Fig. 1C), individual transitions (Fig. 1D, red) were identified as those crossing between boundaries x_1 and x_2 (Fig. 1D, cyan)

defining the barrier region separating the folded and unfolded states (Fig. 1D, orange). Examining individual transition paths for unfolding (Fig. 2A) and refolding (Fig. 2B), their duration was found to vary widely, from less than 10 μ s to over 100 μ s. Moreover, diverse shapes were observed: The speed often varied greatly along the paths, and noticeable pauses occurred at one or more points in the transition. These pauses occurred in the barrier region, providing the first direct visualization of a host of transient, high-energy transition intermediates. In many transitions, the hairpin shuttled back and forth between different extensions, directly demonstrating, at the microscopic level, the diffusive nature of folding.

To test quantitatively the physical picture of folding as a diffusive search, we studied the duration of the transition paths. The transit time for barrier crossing in each transition, t_{tp} , was measured directly from the extension trajectory as the time required to cross from one boundary to the other (Fig. 1D). For consistency, the boundaries were chosen to define the barrier region as the middle half of the total extension change between the folded and unfolded states, Δx_{UF} (20). Measuring transit times individually for 24,591 unfolding transitions and 24,600 refolding transitions, the average value, τ_{tp} , was found to be $27 \pm 2 \mu$ s for unfolding and $28 \pm 2 \mu$ s for refolding (errors represent SEM). These average times were slower than the upper bound for τ_{tp} estimated previously for a much shorter DNA hairpin (8), but similar to the value for an engineered protein

(6). Notably, τ_{tp} was roughly 1000-times smaller than the lifetimes of the unfolded and folded states. As expected from symmetry under time reversal (21), τ_{tp} was the same for both directions.

These results allowed us to test the theory of transition paths experimentally. For example, assuming 1D diffusive motion over a harmonic barrier in the high-barrier limit ($\Delta G^\ddagger > 2 k_B T$), τ_{tp} is related to the diffusion coefficient, D , by

$$\tau_{tp} \approx \frac{\ln(2e^\gamma \beta \Delta G^\ddagger)}{\beta D \kappa_b} \quad (1)$$

where $\Delta G^\ddagger$ is the barrier height, κ_b is the stiffness of the barrier, γ is Euler's constant, and $\beta = 1/k_B T$ is the inverse thermal energy (7, 21). Previously, the measured landscape profile (16) and folding/unfolding rates (15) for hairpin 30R50/T4 were used to calculate D from Kramers' equation for diffusive barrier crossing (22), in turn enabling calculation of τ_{tp} from Eq. 1. These earlier results, $\tau_{tp} = 30 \pm 6 \mu$ s for unfolding and $33 \pm 8 \mu$ s for refolding (10), agree very well with those from the direct measurements, validating Eq. 1. Because τ_{tp} is in principle a more robust measure than approaches that estimate D from rates using Kramers' theory (6, 23), we used Eq. 1 to refine the previous estimate of D . Using the barrier parameters from the reconstructed landscape for this hairpin (16), $\Delta G^\ddagger = 9.1 \pm 0.1 k_B T$ and $\kappa_b = 0.29 \pm 0.02 k_B T/\text{nm}^2$, we found $D = 4.4 \pm 0.4 \times 10^5 \text{ nm}^2/\text{s}$, which is very close to the value of $4.6 \pm 0.5 \times 10^5 \text{ nm}^2/\text{s}$ estimated previously (10).

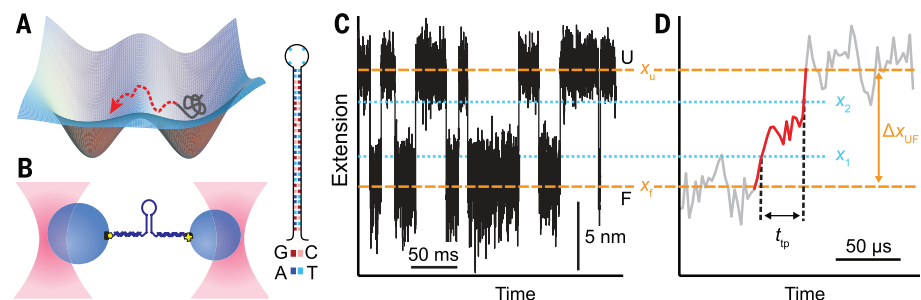
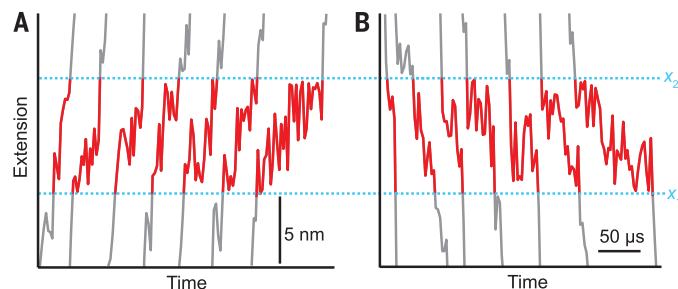


Fig. 1. Transition paths in force spectroscopy measurements. (A) Transition paths represent the brief portions of a folding trajectory spent in crossing the barrier between states (red), in contrast to the majority spent fluctuating within one of the potential wells (gray). (B) A DNA hairpin attached to handles (dark blue) was linked to beads (blue) held in laser traps (pink) applying tension. (Inset) Hairpin sequence. (C) End-to-end extension of a hairpin fluctuating in equilibrium between folded (F) and unfolded (U) states under conditions of constant trap separation. (D) Transition paths were identified as the parts of the trajectories (red) moving between U and F (dashed orange lines). The transit time, t_{tp} , was defined as the time required to cross between the boundaries x_1 and x_2 (dotted cyan lines). Here, the transition shows variations in the speed of the barrier crossing, including a brief pause near the center of the barrier region.

Fig. 2. Transition paths for a DNA hairpin.

Selection of transition paths for (A) unfolding and (B) refolding. Boundaries x_1 and x_2 (cyan) demarcate the barrier region. Transition paths display a wide variety of shapes and transit times.



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We next tested a proposed relationship between τ_{tp} and kinetic rates (21, 24)

$$\tau_{\text{tp}} = \frac{p(\text{TP})}{2k_{\text{U}}P_{\text{F}}} = \frac{p(\text{TP})}{2k_{\text{F}}P_{\text{U}}} \quad (2)$$

where k_{F} and k_{U} are the rates for folding and unfolding, respectively; P_{F} and P_{U} are the equilibrium probabilities to be in the folded or unfolded states; and $p(\text{TP})$ is the fraction of time spent on transition paths. Each of these quantities could be measured directly from the extension trajectories. Comparing the measured τ_{tp} values with the estimates from Eq. 2 based on folding and unfolding rates (Fig. 3A), we found excellent agreement for both folding and unfolding across a range of forces, from the hairpin being mostly folded ($P_{\text{U}} \sim 0.03$) to mostly unfolded ($P_{\text{U}} \sim 0.8$). This agreement validates Eq. 2; furthermore, it emphasizes that the time-reversal symmetry of τ_{tp} holds across the full range of state occupancies.

Even more interesting than the average transit time is the distribution of the individual transit times, because the variability in transit times reflects the fundamentally statistical nature of

the folding process (1). The distribution of transit times, $P_{\text{TP}}(t)$, for unfolding transitions (Fig. 3B, black) had the same shape as that for refolding (Fig. 3B, green), as expected from the time-reversal symmetry of the problem (21). The transit times were broadly distributed, with a peak around 10 μs and a long exponential tail (Fig. 3B, inset). This behavior is similar to that expected for transit across harmonic barriers in the high-barrier limit in the Kramers' regime: $P_{\text{TP}}(t)$ is predicted to have the form

$$P_{\text{TP}}(t) \approx \frac{\omega_{\text{K}} \sqrt{\beta \Delta G^{\ddagger}} \exp[-\beta \Delta G^{\ddagger} \coth(\omega_{\text{K}} t/2)]}{1 - \text{erf} \sqrt{\beta \Delta G^{\ddagger}} \sinh(\omega_{\text{K}} t/2) \sqrt{2\pi \sinh(\omega_{\text{K}} t)}} \quad (3)$$

where $\omega_{\text{K}} = \beta D \kappa_{\text{b}}$ sets the time scale for the decay of the exponential tail (21). The exponential tail of $P_{\text{TP}}(t)$ on its own can also be approximated (21) by

$$P_{\text{TP}}(t) \approx 2\omega_{\text{K}} \beta \Delta G^{\ddagger} \exp(-\omega_{\text{K}} t) \quad (4)$$

Fits of the two distributions to Eq. 3 (Fig. 3B, solid lines) and Eq. 4 (Fig. 3B, dashed lines) were

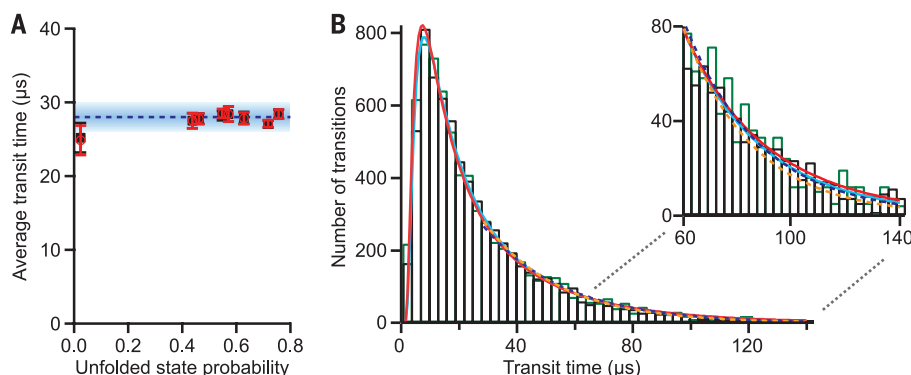


Fig. 3. Transit times for a DNA hairpin. (A) The measured average transition path time (blue) agrees very well with the values predicted by Eq. 2 for folding (red) and unfolding (black) over a range of forces from low (hairpin mostly folded) to high (hairpin mostly unfolded). Error bars represent SEM. (B) The distribution of transit times for barrier crossing is the same for both folding (green) and unfolding (black) transitions. The full distributions are well fit by Eq. 3 (red, unfolding; cyan, folding) and the tails (inset) are separately well fit by Eq. 4 (orange, unfolding; blue, folding), with both fits returning the same results within error.

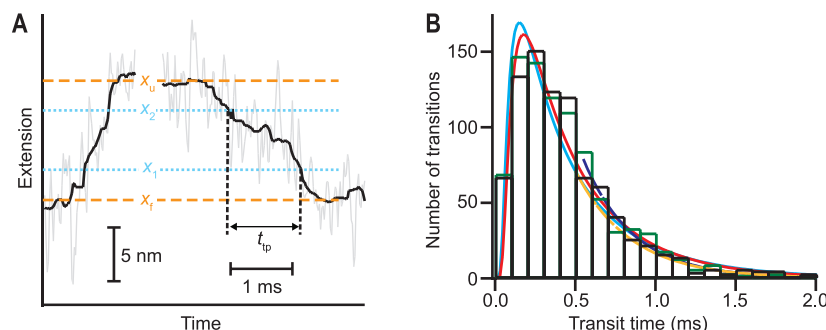


Fig. 4. Transition paths and transit times for a protein. (A) Slow folding of dimeric PrP produced transit times on the millisecond time scale [black, extension trajectories filtered in a 0.4-ms window; gray, unfiltered trajectories; orange, extensions of folded (x_f) and unfolded (x_u) states]. Cyan, boundaries x_1 and x_2 defining the barrier region. (B) The distribution of transit times for barrier crossing is the same for both folding (green) and unfolding (black) transitions. The full distributions are both well fit by Eq. 3 (red, unfolding; cyan, folding) and the exponential tails are separately well fit by Eq. 4 (orange, unfolding; blue, folding).

barely distinguishable, yielding $\omega_{\text{K}} = 6 \pm 3 \times 10^4 \text{ s}^{-1}$ for both folding and unfolding. This result yields $D = 2 \pm 1 \times 10^5 \text{ nm}^2/\text{s}$, which is close to the values calculated from the measured τ_{tp} using Eq. 1 and estimated from rates via Kramers' theory. However, the barrier height returned by the fit, $\Delta G^{\ddagger} \sim 0.4 k_{\text{B}}T$, was much lower than that measured directly from landscape reconstructions (16–19), reflecting the fact that there were more fast transitions than would be expected from the theory for 1D harmonic barriers.

DNA hairpins represent a powerful model system for exploring the physical basis of folding phenomena, but their folding is simpler than that of proteins because they have only secondary structure. We therefore sought to apply the same approach to protein folding. To this end, we focused on a specific structural transition in the prion protein PrP that occurs during the formation of non-native structure in PrP dimers (20). This transition undergoes unusually slow conformational diffusion (25), making it much easier to measure transit times. The transit times were measured directly from extension trajectories, using the same criterion as for the hairpins. Here, however, the transit times were much longer, up to the millisecond scale (Fig. 4A). Once again, the transit times for both unfolding and refolding were broadly distributed with an exponential tail, and the same distribution was observed for both unfolding (Fig. 4B, black) and refolding (Fig. 4B, green).

As expected, τ_{tp} was again the same for unfolding and refolding: $0.5 \pm 0.1 \text{ ms}$ from 1766 transitions. These values also matched (within error) the value expected from Eq. 1, $\tau_{\text{tp}} = 1 \times 10^0 \pm 0.3 \text{ ms}$, which was calculated (25) based on the properties of the energy landscape reconstructed for this transition ($\Delta G^{\ddagger} = 4 \pm 1 k_{\text{B}}T$ and $\kappa_{\text{b}} = 2 \pm 0.5 k_{\text{B}}T/\text{nm}^2$ at $F_{1/2}$) and the diffusion coefficient estimated from the rates using Kramers' theory ($D = 1 \times 10^3 \pm 0.3 \text{ nm}^2/\text{s}$). Fitting the transit time distributions to Eq. 3 (Fig. 4B, solid lines) and Eq. 4 (Fig. 4B, dashed lines), ω_{K} was again similar for both folding and unfolding. The result, $\omega_{\text{K}} = 3 \pm 1 \times 10^3 \text{ s}^{-1}$, implied $D = 1.3 \pm 0.6 \times 10^3 \text{ nm}^2/\text{s}$, in excellent agreement with the result found from the rates and landscape reconstruction using Kramers' theory (25). However, the barrier height returned by the fit, $\Delta G^{\ddagger} = 0.5 \pm 0.3 k_{\text{B}}T$, was once again lower than the measured value, because (as for the hairpin) more short transit times were observed than would be expected from the 1D harmonic theory.

For both molecules, the transit time distributions thus agreed reasonably well with the expectations from 1D harmonic approximations to the previously measured landscapes. The primary discrepancy was a slight bias in the transit time distributions toward shorter times, which caused the fitted barrier height to be lower than the measured height. This bias might arise from a breakdown in the approximations used to derive Eq. 3 (21), such as anharmonicity in the barriers or the need to include higher dimensionality in the landscape, or it could reflect the influence of the dynamics of the beads and handles to which the molecules are tethered (23, 26–28), which were not included in the analysis because they are difficult

to treat (28). The fact that values of the diffusion coefficient (a fundamental descriptor of the dynamics) obtained from different experimental variables using 1D theories are similar suggests that these 1D descriptions of folding (8, 11, 14, 19, 29) can hold even at the microscopic level, despite their many simplifying assumptions.

The ability to observe and characterize transition paths opens up many exciting avenues to explore in folding studies by allowing more direct investigation of transition states and the microscopic thermally driven motions that underlie the conformational search. Previously invisible microstates along the transition paths may now be detectable, permitting their properties to be characterized directly. Moreover, it may be possible to distinguish different classes of transition paths having different properties, such as barrier heights, intermediates, or roughness. The potential for deeper integration of experiment and simulation through direct comparisons of the transition path properties found experimentally to the results of atomistic simulations is also exciting (4). Because the transit time is so sensitive to the diffusion coefficient D (4, 6, 23), such measurements also hold great promise for investigating the effects of solvent viscosity and internal friction (4, 6, 30).

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Fig. S1

References (31–36)

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CANCER

SCS macrophages suppress melanoma by restricting tumor-derived vesicle-B cell interactions

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Tumor-derived extracellular vesicles (tEVs) are important signals in tumor–host cell communication, yet it remains unclear how endogenously produced tEVs affect the host in different areas of the body. We combined imaging and genetic analysis to track melanoma-derived vesicles at organismal, cellular, and molecular scales to show that endogenous tEVs efficiently disseminate via lymphatics and preferentially bind subcapsular sinus (SCS) CD169⁺ macrophages in tumor-draining lymph nodes (tdLNs) in mice and humans. The CD169⁺ macrophage layer physically blocks tEV dissemination but is undermined during tumor progression and by therapeutic agents. A disrupted SCS macrophage barrier enables tEVs to enter the lymph node cortex, interact with B cells, and foster tumor-promoting humoral immunity. Thus, CD169⁺ macrophages may act as tumor suppressors by containing tEV spread and ensuing cancer-enhancing immunity.

Although cancer is driven by tumor cell–endogenous genetic mutations, it is also modulated by tumor cell–exogenous interactions with host components, including immune cells (1). Tumor-induced host immune system activation can occur both within and away from the tumor stroma and may involve different communication signals, including soluble factors (2) and tumor-derived extracellular vesicles (tEVs) (3). tEVs are key candidate conveyors of information between cancer and host im-

mune cells because they can travel long distances in the body without their contents degrading or diluting. tEVs may transfer surface receptors or intracellular material to different host acceptor cells (4–6); these processes have all been associated with altered antitumor immunity and enhanced cancer progression (7). Circulating tEVs also have diagnostic and prognostic potential, as they can be used to detect early cancer stages (8) and to predict overall patient survival (4) and treatment responses (9). Despite increased understanding of tEVs' importance, a critical barrier to progress in the field has been our limited ability to assess the impact of vesicles that are produced in vivo (7). To shift current experimental research on tEV–host cell interactions, we combined imaging and genetic approaches to track endogenously produced tEVs and their targets at different resolutions and scales.

We assessed the whole-body biodistribution of tumor-derived material in mice bearing genetically modified B16F10 melanoma tumors (B16F10-mGLuc), which produce tEVs carrying membrane-bound *Gaussia* luciferase (mGLuc) (10) (fig. S1). Quantification of tEV-bound mGLuc activity in various

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Fig. 1. Endogenous tEVs disseminate via lymph and interact with tumor-draining LN SCS macrophages. (A) Relative mGLuc luminescence activity (per microgram of tissue) in various organs isolated from mice carrying B16F10-mGLuc⁺ melanoma tumors on week 2 after tumor challenge (two independent experiments, $n = 8$ to 10). (B to E) Quantification of host dLNGFR⁺ cells in (B) total tdLN and ndLN cells, (C) lymphoid/myeloid cell fractions, and (D) macrophage subsets isolated from mice carrying dLNGFR⁺ B16F10 melanoma tumors on week 2 after tumor challenge (two independent experiments, $n > 10$). (E) Representative multiphoton micrographs of an explanted tdLN from a mouse carrying CD63-eGFP⁺ B16F10 melanoma on week 2 after tumor challenge (two independent experiments, $n = 6$). (F) Experimental outline of lymph collection (left) and quantification of mGLuc signal in cell-free lymph and cells from lymph (two independent experiments; $n = 11$). ** $P < 0.01$. **** $P < 0.0001$ (Mann-Whitney test). Lum, luminescence; Mø, macrophage; MS, medullary sinus; ndLN, non-tumor-draining LN; TAM, tumor-associated macrophages; tdLN, tumor-draining LN.

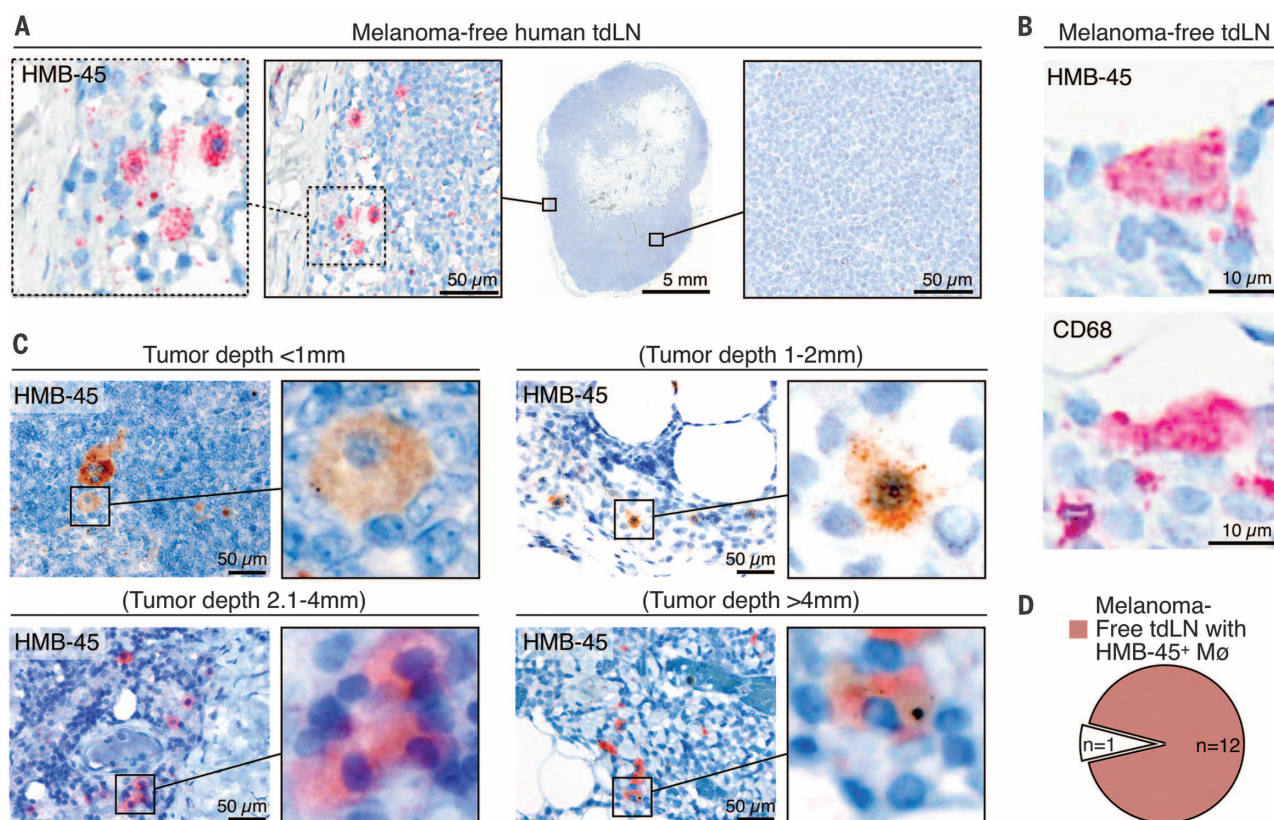
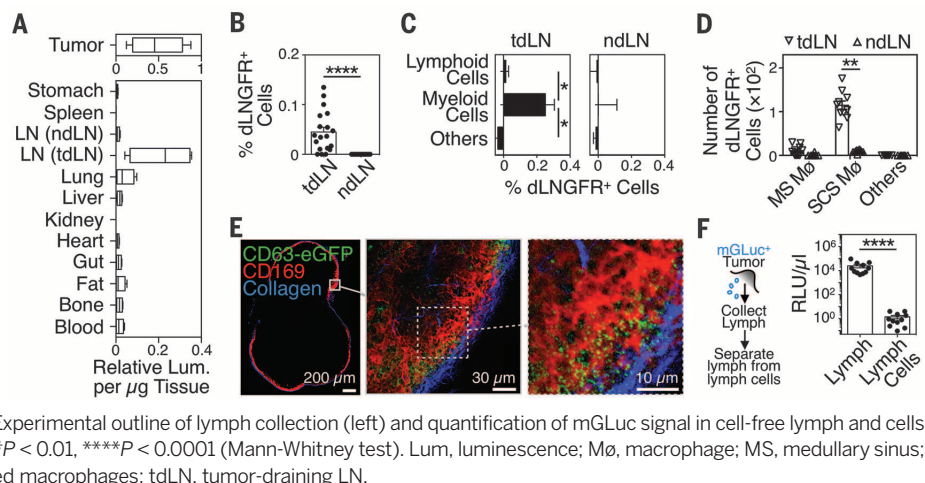


Fig. 2. Human SCS macrophages collect tumor-derived materials in melanoma-free tumor-draining LNs. (A) Immunohistochemistry for the melanoma marker HMB-45 (red) in a tdLN from a melanoma-free (i.e., stage N0) patient. The tissue was counterstained with hematoxylin (blue). (B) Immunohistochemistry for HMB-45 melanoma (top) and CD68 macrophage markers (bottom) in sequential sections from a melanoma-free (i.e., stage N0) tdLN.

(C) HMB-45 immunohistochemistry (brown or red) in tdLNs from melanoma-free patients with different tumor stages (according to American Joint Committee on Cancer guidelines). Primary tumor depth is indicated above each image. Tissues were counterstained with hematoxylin (blue). (D) Pie chart illustrating the fraction of patients containing HMB-45⁺ macrophages in melanoma-free tdLNs.

tissues from B16F10-mGLuc⁺ tumor-bearing mice not only confirmed that B16F10-mGLuc⁺-derived tEVs can exit the tumor stroma and relocate to remote organs but also identified the highest relative mGLuc activity in tumor-draining lymph nodes (tdLNs) when compared to blood, spleen, bone, lung, liver, non-tumor-draining LNs (ndLNs), and other tissues (Fig. 1A and fig. S2A). Consistently,

we measured higher mGLuc signal in lymph than in plasma (fig. S2B). Control tumors expressing secreted *Gaussia* luciferase (sGLuc) did not generate bioluminescence activity in tdLNs (fig. S2C).

To decipher endogenous tEVs' interactions in tdLNs at the cellular level, we investigated mice bearing genetically modified B16F10 melanoma cells expressing two membrane-bound reporters:

the vesicular membrane-associated protein CD63 fused with enhanced green fluorescent protein (CD63-eGFP), and the ubiquitous transmembrane marker dLNGFR (truncated receptor for nerve growth factor) (fig. S3). Flow cytometry-based analyses revealed dLNGFR⁺ cells in tdLNs but not in ndLNs (Fig. 1B). These tdLNs did not include tumor cells or tumor cell apoptotic bodies

(figs. S4 to S6). The dLNGFR signal originated mostly from myeloid cells, not lymphoid cells (Fig. 1C). Among tdLN myeloid cells, the CD11b⁺ side scatter-low fraction, which resembles sub-capsular sinus (SCS) macrophages (12), was dLNGFR⁺, whereas CD11b⁺ SSC^{HI} marginal sinus macrophages remained largely dLNGFR⁻ (Fig. 1D and fig. S7). Multiphoton microscopy and three-dimensional reconstructions of tEV distribution confirmed CD169⁺ SCS macrophages as a major host cell type interacting with CD63-eGFP⁺ tEVs in vivo (Fig. 1E and figs. S8 and S9). The vesicles accumulated principally between 10 and 20 μ m below the LN capsule and adjacent to CD169⁺ SCS macrophages, which occupy the space between 20 and 80 μ m below the capsule.

We asked whether CD169⁺ SCS macrophages originate from the tumor stroma, where they may initially capture tEVs. B16F10 tumors were implanted in mice ubiquitously expressing the photoconvertible protein Kaede (12), and the tumor site was exposed to ultraviolet light in order to shift Kaede fluorescence emission from green to red selectively in tumor-infiltrating host cells (fig. S10, A and B). The tdLN SCS macrophages remained green 24 hours later and therefore did not origi-

nate from the tumor stroma (fig. S10C). Photoconverted cells in tdLNs were mostly CD103⁺ DCs (fig. S10D). These migratory cells might not be involved in carrying tEVs to LNs, because analysis of lymph collected from B16F10-mGLuc tumor-bearing mice revealed mGLuc activity that was higher by a factor of $>10^4$ in cell-free fractions than in cells from lymph (Fig. 1F). These data suggest that tEVs freely disseminate to tdLNs, where they preferentially bind resident SCS macrophages.

To define our findings' relevance for human disease, we examined cancer-free sentinel LN (CF-SLN) biopsies from 13 melanoma patients (table S1). Melanin pigment staining was found selectively in macrophage-like populations (figs. S11 and S12, A to C). We then assessed melanoma-derived material by staining CF-SLNs with the monoclonal antibody (mAb) clone HMB-45, which is used to pathologically evaluate melanoma metastasis in regional SLNs. HMB-45 reacts with a transmembrane glycoprotein that is part of the gp100 pre-melanosome complex and is expressed by $>80\%$ of melanomas (13). Although the SLNs analyzed were melanoma-free (i.e., stage N0), we identified HMB-45⁺ cells that corresponded to macrophages morphologically and resided mostly

near the LN capsule (Fig. 2A and fig. S12D). Serial staining of CF-SLN sections for HMB-45 and the macrophage marker CD68 confirmed that the observed HMB-45⁺ cells were CD68⁺ macrophages (Fig. 2B and fig. S13). To interrogate the temporal course of HMB-45⁺ signal appearance during melanoma progression, we assessed CF-SLNs from patients with distinct clinical stages based on Breslow's thickness (tumor depths ranging from <1 mm to >4 mm). We identified HMB-45⁺ macrophages in $>90\%$ of patients independent of tumor progression (Fig. 2, C and D), which suggests that melanoma-derived material reaches SLNs early in cancer progression, similar to our observations in mice (fig. S14).

Given that EVs can deliver intracellular RNAs and proteins into target cells and that horizontal transfer can shape the fate of acceptor cells (4–6), we asked whether such transfer characterizes SCS macrophage–tEV interactions. We used transgenic mice that express yellow fluorescent protein (YFP) upon Cre-mediated recombination and challenged these mice with genetically modified B16F10 melanoma tumor cells expressing Cre (fig. S15, A to E). Fusion of Cre⁺ tEVs with host acceptor cells would irreversibly induce YFP

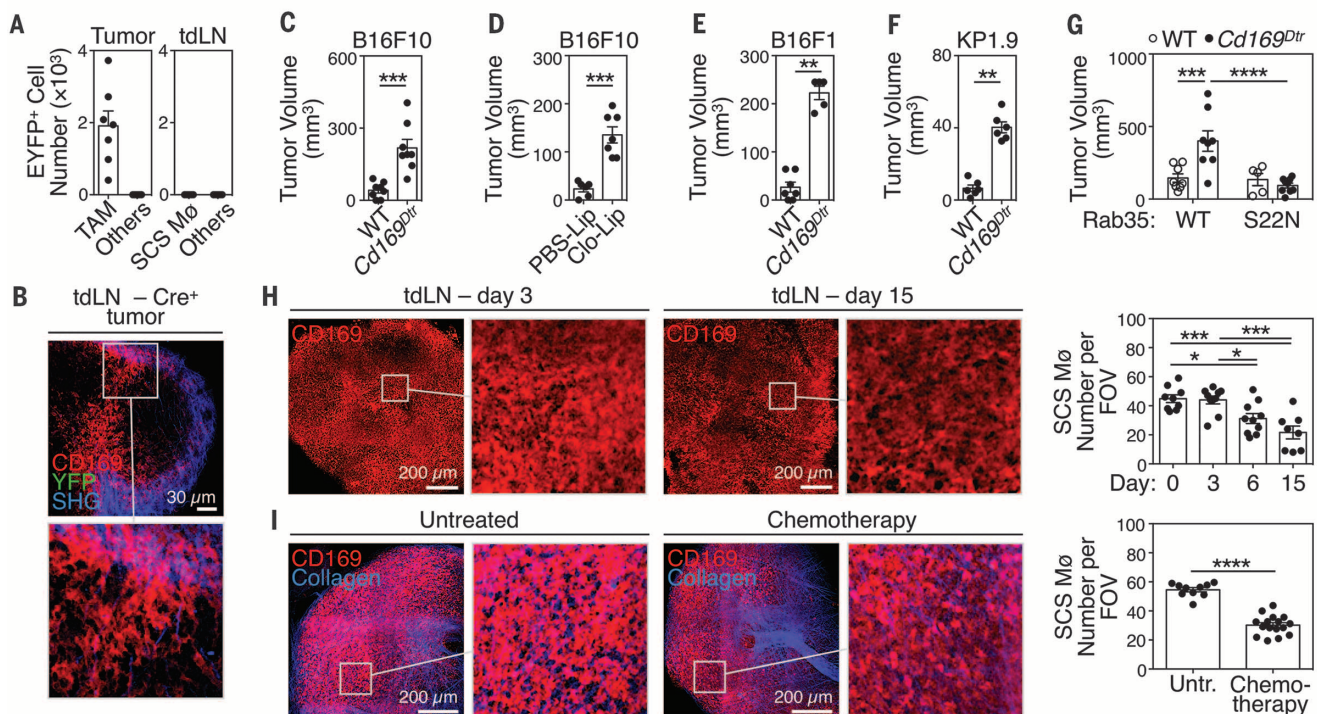


Fig. 3. SCS macrophage–tEV interactions suppress tumor growth. (A) Number of eYFP⁺ TAMs, SCS macrophages, and other cells on week 2 after challenging Cre-reporter mice with Cre⁺ B16F10 tumors (two independent experiments, $n = 7$). (B) Multiphoton micrographs of LNs draining Cre⁺ tumors (one experiment, $n = 3$). (C) B16F10 tumor volume in wild-type or *Cd169^{Dtr/Wt}* mice, all treated with DT intraperitoneally (i.p.; two independent experiments, $n = 8$). (D) B16F10 tumor volume in wild-type mice treated with phosphate-buffered saline (PBS)–Lip or Clo-Lip subcutaneously (s.c.; two independent experiments; $n = 6$ or 7). (E) B16F1 melanoma tumor volume in wild-type or *Cd169^{Dtr/Wt}* mice, all treated with DT i.p. ($n = 5$ to 7). (F) KP1.9 lung adenocarcinoma tumor volume in wild-type or *Cd169^{Dtr/Wt}* mice, all treated with DT i.p. ($n = 6$). (G) B16F10 tumor volume in wild-type or *Cd169^{Dtr/Wt}* mice, all treated with DT i.p., and

challenged with tumors expressing either Rab35^{WT} or Rab35^{S22N} ($n = 5$ to 8). (H) Left: multiphoton micrographs (2D projections of 30 high-resolution optical sections spanning the whole LN with 2 μ m Z-spacing) of tdLNs on day 3 and 15 after B16F10 tumor challenge ($n = 2$ or 3). Right: Quantification of SCS macrophage barrier disruption measured as CD169⁺ SCS macrophage number per field of view. (I) Left: Multiphoton micrographs [obtained similarly as in (H)] of inguinal LNs 1 week after starting i.p. paclitaxel/carboplatin injections, 3 times per week ($n = 4$). Right: quantification as in (H). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ [Mann-Whitney test for (C), (D), (E), (F), and (I); two-way analysis of variance (ANOVA) for (G); one-way ANOVA with Tukey's multiple comparisons test for (H)]. Clo-Lip, clodronate-loaded liposomes; DT, diphtheria toxin; FOV, field of view; PBS-Lip, PBS-loaded liposomes; Untr., untreated.

expression in the target cell. Analysis of B16F10-Cre⁺ tumor-bearing mice identified Cre-induced YFP expression in tumor-associated macrophages (TAMs); however, tdLN CD169⁺ SCS macrophages (and all other tdLN cells) remained exclusively YFP⁺ (Fig. 3, A and B, and fig. S15F). Thus, on their own, endogenous tEVs are unlikely to modulate SCS macrophages through horizontal transfer.

Because SCS macrophage-tEV interactions may regulate tumor progression independently from horizontal transfer, we assessed whether modulating SCS macrophages and/or tEVs affects cancer growth in vivo. We examined *Cd169*^{Dtr/Wt} knock-in mice in which CD169⁺ LN macrophages were specifically depleted by diphtheria toxin (DT) injection (figs. S16 and S17). CD169⁺ LN macrophage removal significantly enhanced B16F10 tumor growth (Fig. 3C). Similarly, specifically depleting CD169⁺ LN macrophages by subcutaneous administration of clodronate liposomes (figs. S18 and S19) accelerated B16F10 tumor progression in wild-type mice (Fig. 3D). Thus, CD169⁺ LN macrophages act as “tumor suppressors” in orthotopic B16F10 melanoma; these findings were extended to orthotopic B16F1 melanoma (Fig. 3E) and KP lung adenocarcinoma (Fig. 3F).

To assess whether interactions between endogenous tEVs and SCS macrophages affect tumor progression, we introduced a single copy of either Rab35 wild-type (Rab35^{WT}) or Rab35 dominant-negative Ser²² → Asn mutant (Rab35^{S22N}) (14) into B16F10 melanoma cells. These tumors had normal or impaired capacity, respectively, to release tEVs (fig. S20). As expected, removing CD169⁺ macrophages accelerated B16F10 Rab35^{WT} tumor progression; however, Rab35^{S22N} tumors grew similarly with or without CD169⁺ macrophages (Fig. 3G). The observation that enhanced tumor growth from SCS macrophage ablation only occurs in the context of sufficient tEV production supports a causal link between SCS macrophage-tEV interaction and tumor growth.

We then asked whether cancer disrupts SCS macrophage network organization, because pathogens entering LNs can induce such alterations (15). Three-dimensional multiphoton imaging of tdLNs revealed a decrease in CD169⁺ SCS macrophage density as soon as day 6 after tumor challenge (Fig. 3H). This may happen because tdLNs enlarge without expanding their SCS macrophage pool, as indicated by photoconversion (fig. S10), parabiosis (fig. S21A), 5-bromo-2'-deoxyuridine (BrdU; fig. S21B), and Ki67 labeling studies (fig.

S21C). These results imply different ontogenesis for TAMs and SCS macrophages because, unlike SCS macrophages, most TAMs derive from circulating monocytes and can divide in some tumors (16, 17). Chemotherapy with paclitaxel and carboplatin (Fig. 3I) and immunotherapy with a small-molecule CSF-1R inhibitor (fig. S22) also reduced CD169⁺ SCS macrophage density. Thus, the SCS macrophage barrier can be disrupted both during the natural course of tumor progression and upon anticancer treatment.

Because SCS macrophage-tEV interactions are geographically restricted to tdLNs, yet modulate the outgrowth of distant tumors, they may influence a systemic response to cancer. Indeed, depletion of CD169⁺ tdLN macrophages on one side only (fig. S23) was sufficient to accelerate both contralateral and ipsilateral tumor growth (Fig. 4A). We hypothesized that tEVs may bind and regulate discrete host components upon disruption of the SCS macrophage layer. Interestingly, tdLN multiphoton imaging revealed that, without SCS macrophages, tEVs efficiently penetrated the LN cortex (Fig. 4B). These findings indicate that SCS macrophages act as tEV gatekeepers—a capacity that resembles these macrophages' ability to prevent the systemic spread of lymph-borne pathogens (15, 18–20).

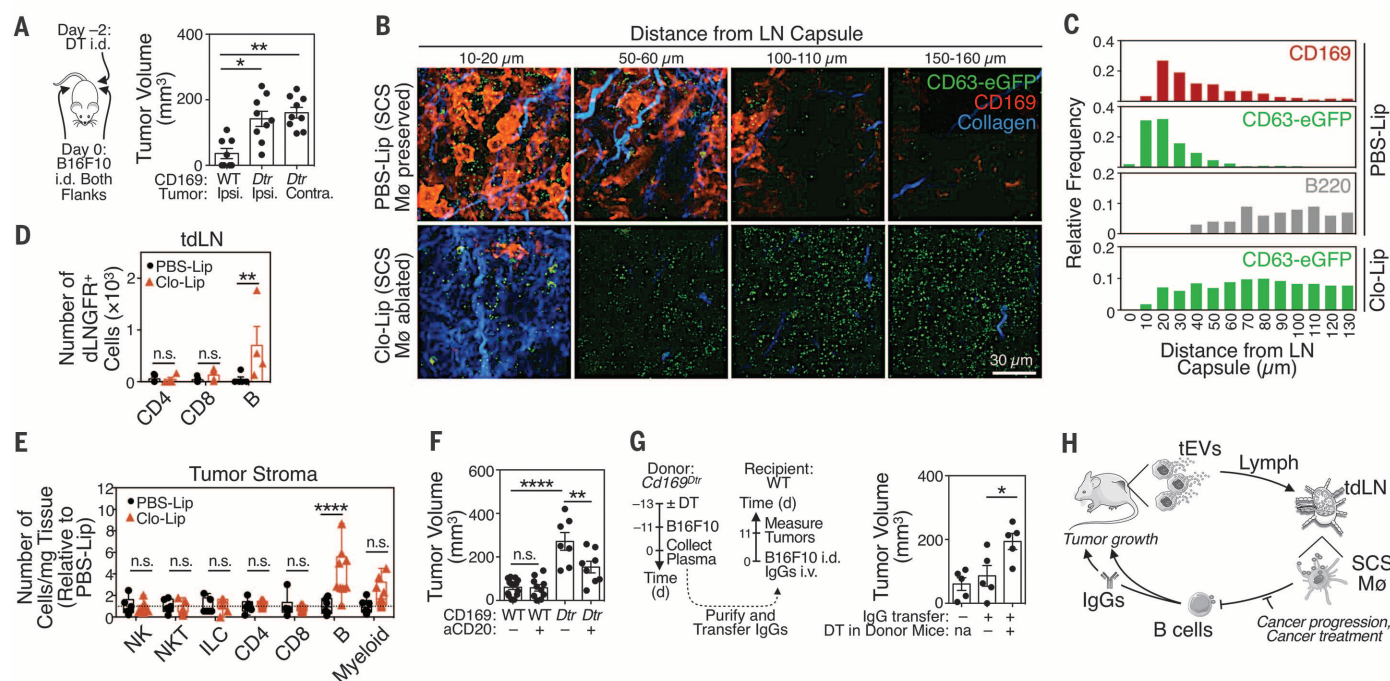


Fig. 4. SCS macrophage-tEV interactions suppress tumor-promoting B cell immunity. (A) Tumor volumes in *Cd169*^{Dtr/Wt} mice locally treated with DT intradermally (i.d.) on one side and challenged with B16F10 tumors on both flanks i.d. ($n = 9$). (B) Multiphoton micrographs of tdLNs from CD63-eGFP⁺ B16F10 tumor-bearing mice (treated with PBS-Lip or Clo-Lip s.c.) and imaged at the indicated depth below the LN capsule (blue). Red, CD169; green, eGFP (two independent experiments, $n = 3$). (C) Distance between the indicated entities (CD169⁺ cells, CD63-eGFP⁺ tEVs, and B220⁺ cells) and the LN capsule, plotted as relative frequency versus position. (D) Flow cytometry-based quantification of dLNGFR⁺ lymphocyte subsets in tdLNs from dLNGFR⁺ B16F10 melanoma-bearing mice treated with PBS-Lip or Clo-Lip s.c. ($n = 4$ or 5). (E) Flow cytometry-based quantification of different cell types in B16F10

tumors from mice treated with PBS-Lip or Clo-Lip (data are normalized to PBS-Lip-treated mice; two independent experiments, $n = 9$). (F) B16F10 tumor volumes (day 9) in wild-type and *Cd169*^{Dtr/Wt} mice treated with DT i.p. and/or with CD20-depleting mAb ($n = 7$ to 10). (G) B16F10 tumor volumes in wild-type recipient mice that received IgGs (25 µg) isolated from plasma of *Cd169*^{Dtr/Wt} donor mice treated with PBS or DT i.p. ($n = 5$). Mice that did not receive IgGs were used as controls. (H) Proposed model. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$; n.s., not significant. Kruskal-Wallis test with Dunn's multiple comparisons test for (A); two-way ANOVA with Sidak's multiple comparisons test for (D) and (E); one-way ANOVA with Tukey's multiple comparisons test for (F) and (G). aCD20, CD20 mAb; Contra, contralateral; DTR, DT receptor; ILC, innate lymphoid cells; Ipsi, ipsilateral; NK, natural killer cells; NKT, natural killer T cells.

Multiphoton imaging of tdLNs in SCS macrophage-depleted mice further revealed that tEVs reached B cell follicles (Fig. 4C). Also, flow cytometry-based analysis of tdLNs identified B cells as the only detectable immune population physically interacting with tEVs in these mice (Fig. 4D and fig. S24A). Such interaction was lost in mice bearing Rab35S22N tumors, which are impaired to secrete tEVs (fig. S24B). B cells remained YFP⁺ in tdLNs from B16F10-Cre⁺ tumor-bearing Cre-reporter mice treated with clodronate liposomes, indicating that tEV horizontal gene transfer to B cells does not occur in the absence of SCS macrophages (fig. S24A). However, various B cell subsets increased in tdLNs, and concomitantly decreased in ndLNs, as tumors progressed (fig. S24, C and D). The concentration of tumor-infiltrating B cells also increased by a factor of ~3 in SCS macrophage-depleted mice, whereas other immune cell populations remained detectably unchanged (Fig. 4E and fig. S25). To test a causal role for B cells in enhancing melanoma growth after CD169⁺ LN macrophage ablation, we removed B cells by means of a CD20 mAb in DT-treated *Cd169^{Dtr/Wt}* mice. B cell ablation significantly decreased tumor progression in this experimental setting (Fig. 4F). These data imply that B cells are tumor-promoting cells through tEV-B cell interactions that can be suppressed by SCS macrophages.

Because B cells may foster tumor progression by producing autoantibodies (21–23), we tested whether manipulating SCS macrophages modulates immunoglobulin G (IgG) responses. Indeed, CD169⁺ LN macrophage depletion amplified tdLN plasma cells (fig. S26A) and increased both plasma IgG concentration (fig. S26B) and IgG affinity for tumor antigens (fig. S26C). The increased IgG concentration required full-fledged tEV secretion by tumors (fig. S26D). Most important, transfer of circulating IgGs from B16F10 tumor-bearing mice, in which SCS macrophages were depleted, significantly accelerated tumor growth in SCS macrophage-competent mice (Fig. 4G and fig. S27). Thus, SCS macrophages can suppress cancer progression at

least partly by limiting pro-tumor IgG responses (Fig. 4H).

Our study identifies SCS macrophages as tumor-suppressive cells, in contrast to TAMs that often display tumor-promoting activities (24). Yet tumor progression and at least some therapeutic agents undermine the SCS macrophage barrier, thereby enabling tEV interaction with B cells in the LN cortex and activating tumor-enhancing B cell immunity. Previous studies that investigated acute responses to pathogens and model foreign antigens had established that SCS macrophages can promote B cell responses (15, 20, 25–27). The present data suggest that SCS macrophages can also provide a physical barrier to B cell activity under specific circumstances. It is possible that SCS macrophages acquire different functions when exposed continuously to inflammatory triggers or in the context of sterile inflammation. Additionally, tEVs may have unique properties that prevent their presentation by SCS macrophages to B cells or that alter SCS macrophage functions *in vivo*. Thus far, macrophage-targeting therapies to treat cancer are mostly aimed at depleting these cells indiscriminately (28). Instead, our results favor therapeutic approaches that limit harmful TAM functions while leaving SCS macrophages unaffected. Whether it is possible to selectively expand SCS macrophages to control cancer also deserves consideration. In support of this scenario, a high density of CD169⁺ macrophages in regional LNs positively correlated with longer overall survival in patients with colorectal carcinoma (29).

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/352/6282/242/suppl/DC1
Materials and Methods
Figs. S1 to S27
Table S1
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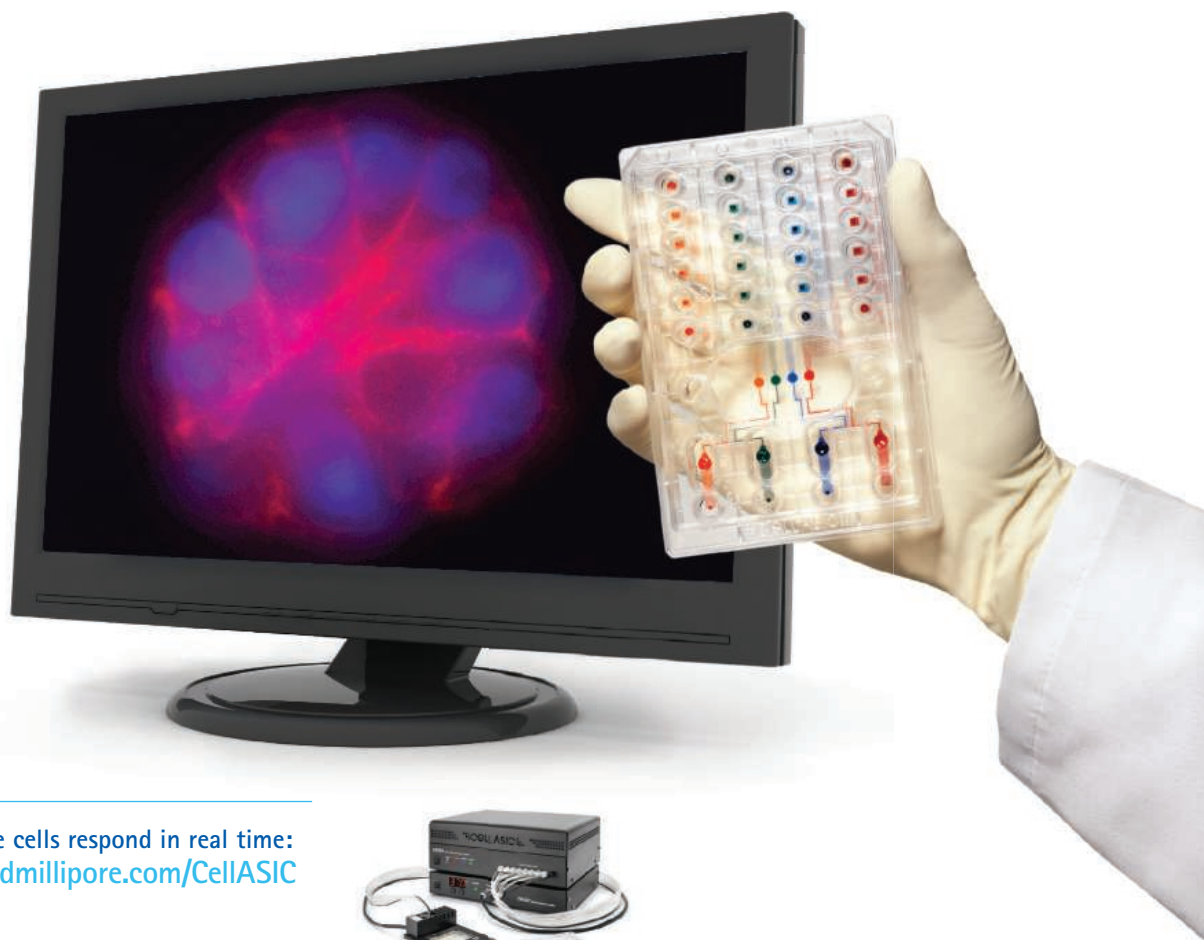
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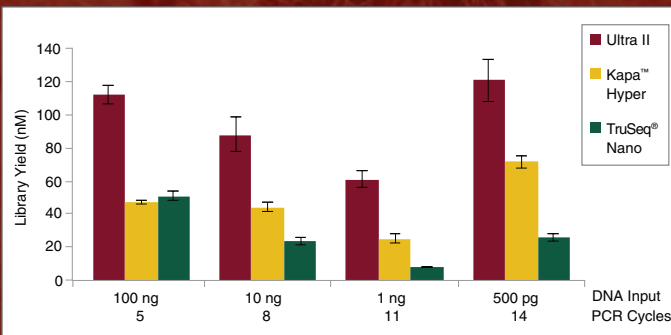
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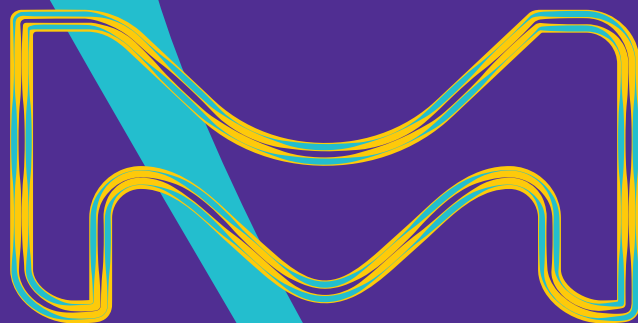
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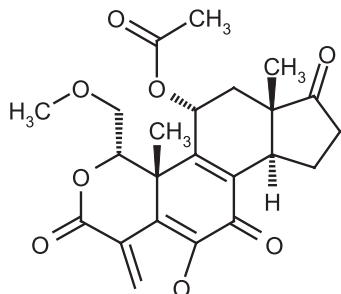


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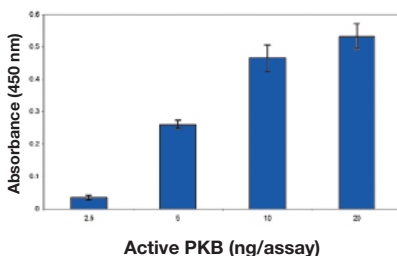
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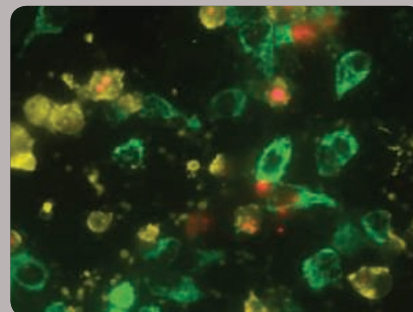
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Quantitative data from large populations with single-cell precision

Flow cytometry addresses this need for quantitative data from significant cell populations by interrogating individual cells for the presence and relative strength of signal from fluorescent reagents or antibodies. However, traditional flow cytometers require extensive operator training and expertise, and sheath fluid-based systems are characterized by extensive setup and shutdown—as well as considerable cost to purchase, operate and maintain.

The Muse® Cell Analyzer was developed to give researchers simple, affordable access to the quantitative data that flow cytometry provides for measuring markers of viability, mitochondrial health, protease activity, and more. Built on flow cytometry principles, Muse® uses microcapillary fluidics and pre-optimized reagents to create an inexpensive, compact, portable system that requires little setup and no expertise to operate. These attributes present a rapid, simplified alternative to more time-consuming methods like Western blot (that may also demand considerable technical expertise) for routine analysis of cell culture health, and to assess the effects of compounds for toxicology and drug discovery screening.

Assay	Key materials and equipment needed	Hands-on time	Typical total assay time: From cells to data
ELISA	Coated multiwell plates	1.5 hours	8 hours – 2.5 days
	Capture antibody		
	Detection antibody conjugate		
	Enzyme substrate		
	Stop solution		
	Plate washer		
Dot blot	ELISA reader	1.25 hours	5.5 hours
	Blot cassette		
	Blocking buffer		
	Nitrocellulose membrane		
	Protein standard		
	Primary antibody		
Traditional Western blot protocols **	Secondary antibody	2 – 3 hrs	10 hrs – 2.5 days*
	Detection reagent		
	Lysis buffer protein quantification kit		
	Protein standards		
	SDS page gels		
	Electrophoresis chamber		
	Loading buffer		
	Running buffer		
	Transfer buffer		
	Protein transfer chamber		
	Membranes		
	Filter paper		
Muse®	Muse® Cell Analyzer	10-15 mins	10 mins – 4 hrs.
	Relevant Muse® assay		

Table 1. Workflow comparison among protein detection methods. For proteins not already in solution, bulk methods require lysis of samples to render proteins accessible to detection. Reagents, supplies and equipment for separation, transfer, probing, detection and imaging may be needed, depending on technique. Lengthy primary antibody incubations and serial washes following each binding step can result in significant hands-on and total elapsed time for routine protocols. The Muse® Cell Analyzer uses preoptimized reagent cocktails to minimize variation and instrument setup, resulting in appreciable reductions in time spent on the bench and total start-to-result time.

*Significant time savings can be achieved by use of a vacuum-driven method such as the SNAP i.d.® 2.0 system for Western blotting.

SUMMARY OF PROTOCOL

Culture cells, including negative and positive controls, for time needed to induce apoptosis.



Dilute Muse® 10X Caspase Buffer to 1X with DI water.



Prepare cell samples in 1X Caspase Buffer for incubation with Muse® MultiCaspase Reagent working solution.



Reconstitute Muse® MultiCaspase Reagent with 50 µL of DMSO to make stock solution.



Dilute Muse® MultiCaspase Reagent stock solution 1:160 with 1X PBS to make working solution.



Prepare Muse® Caspase 7-AAD working solution by adding 2 µL of 7-AAD to 148 µL of 1X Caspase Buffer.

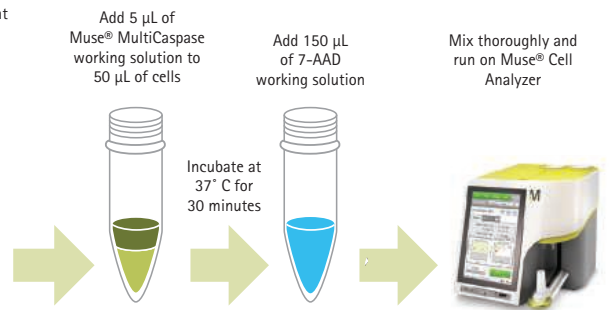


Figure 1. Typical Muse® assay experimental protocol summary. Steps shown are from the protocol for the Muse® MultiCaspase Assay kit (Cat. No. MCH100109) for detection of the activity of caspases 1, 3, 4, 5, 6, 7, 8, and 9. Results from this assay are available in approximately one hour, about 20 minutes of which requires hands-on activity.

Individual cell analysis for protein expression and cell quantitative alternative to standard bulk detection methods

Detection and quantitation of proteins in biological samples is an essential task routinely performed in countless disciplines and in laboratories all over the world, for activities ranging from basic protein characterization to clinical diagnostic testing and drug development. Common methods for protein detection include enzyme-linked immunosorbent assay (ELISA), dot blot, and Western blot (also called protein immunoblot).

While these standard assays continue to present a reliable means of cell analysis for thousands of targets, recent advances in instrumentation offer significant improvements in time savings and convenience for common markers. An additional limitation of bulk assays is the need to homogenize cells or tissue, resulting in a loss of information from individual cells in a population. Techniques that detect and report signal from individual cells can provide quantitative data from large populations about protein target or other marker levels, from cells of varying phenotype, developmental state, or health status.

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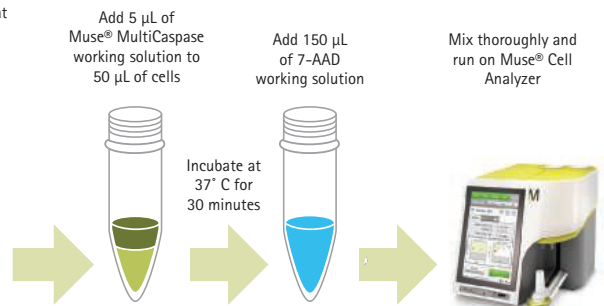


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health markers provides a rapid, methods for fundamental assays

Population means vs. individual cell quantitation

Data that can be quantified are increasingly important in the life sciences, as quantitative data are objective and therefore considered more reliable, as well as being subject to statistical analysis. Quantitative data are assumed to be more representative of populations than qualitative data, and therefore must be characterized both by significant sample size and by the capacity to measure individual events in a sample.

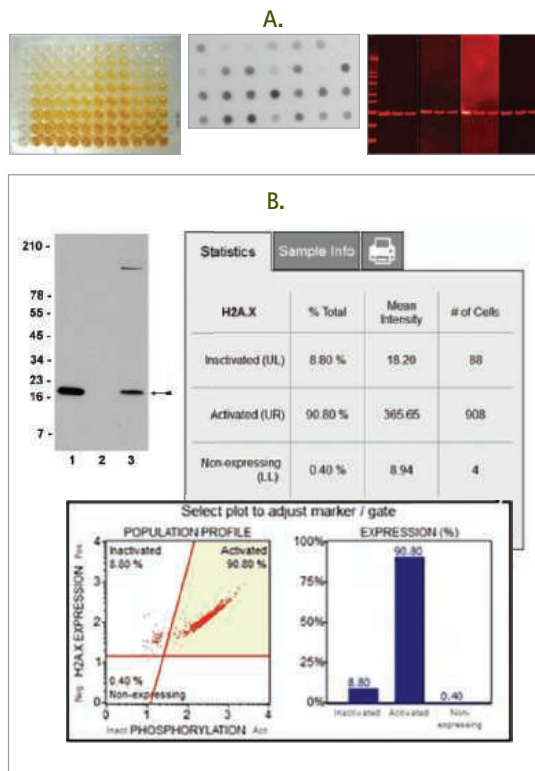


Figure 2. Common bulk immunodetection assay results contrasted with quantitative data. A. Enzyme-linked immunosorbent assay (ELISA), left panel, uses a colorimetric or a fluorescent detection reagent. Spectrophotometry can be used to transform signal intensity into numerical values, but signal intensity is a mean from all cells or cell products in a sample, as is the case with dot blot (middle panel). The right panel is an example of fluorescent detection of immunoblot (Western blot), showing the 'ladder', or molecular standard, in lane 1. B. Left panel, Western blot of recombinant histone H2A.X (lane 1), recombinant histone H2A (lane 2), and acid extracted proteins from HeLa cells (lane 3) were probed with anti-histone H2A.X. The right panel shows representative data from the Muse® H2A.X activation dual detection assay, which uses two directly conjugated antibodies against the unmodified and phosphorylated histone target to map signal from every cell in the sample onto a scatter plot. Absolute numbers and percent of cells activated in the sample are automatically calculated and displayed on the 'Statistics' tab.

The Muse® software automatically returns cell-by-cell results from these reagents, unlike microscopy or other low-throughput, time-consuming or subjective techniques for measuring their signal. Muse® assays are selected to provide an efficient means for the most essential viability, cell health, and signaling screening, and reagents are pre-optimized to minimize variation and the need for complex setup adjustments that characterize traditional open-system cytometers.

Despite its small size and remarkably simplified operation, the Muse® system returns the same powerful single-cell data as larger, more costly and complex systems. The availability of rapid, quantitative cell analysis without the need for extensive investment in supplies or trained personnel has the potential for significant impact on compound screening and cell culture model paradigms in the pharmaceutical and life science research domains.

To learn more about the Muse® Cell Analyzer and see a complete list of Muse® assays, please visit: www.emdmillipore.com/muse



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Although spectrophotometry and densitometry can be used to transform sample well color or the size of a blot or band into numerical values for comparison of relative signal intensity among samples, these methods rely on homogenization of all of the cells or tissue in a particular sample. Western blot relies on concurrent electrophoresis of a mixture of proteins of known weight to create a standard, or 'ladder' of bands on the blot, to which positive bands from sample lanes are compared for confirmation of the protein's identity (Figure 2A, right panel). Because no identity information can be gained from immunoblot without the standard for comparison, Western blot is considered 'semi-quantitative'.

Conclusions

Immunoblot and immunosorbent assays continue to be among the most popular methods for protein detection in the life sciences, as they are amenable to measuring virtually any target for which an epitope binder such as an antibody can be developed. These methods are constrained, however, by the inability to capture population variation due to the homogenization of sample. Standard bulk methods may also not be optimal for routine screening because of the time they consume in the lab and the expertise they require in order to optimize reagents and to obtain, interpret, and troubleshoot results.

Simplified flow cytometry-based analysis presents a rapid, uncomplicated, cell-based alternative to methods such as immunoblot, particularly for routine and frequent screening of cell cultures, or for response of cell models to compounds in development for chemotherapeutics, drug discovery, cosmetics and similar applications. In addition to detecting key protein targets, The Muse® system incorporates assays for detection using familiar cell status indicators that do not rely on antibody-protein interactions, such as fluorescent membrane integrity dyes and nucleic acid binders.

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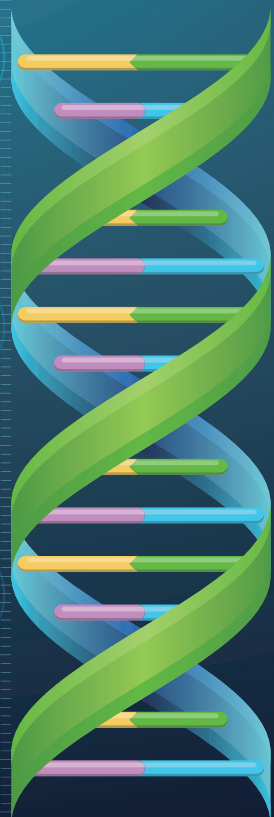
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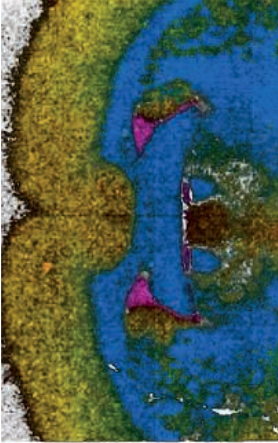
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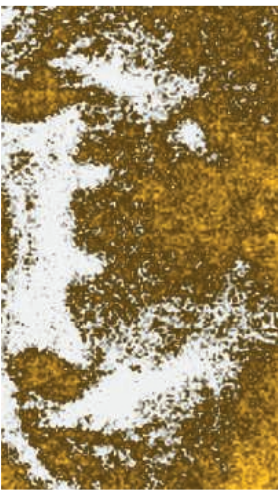
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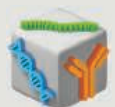
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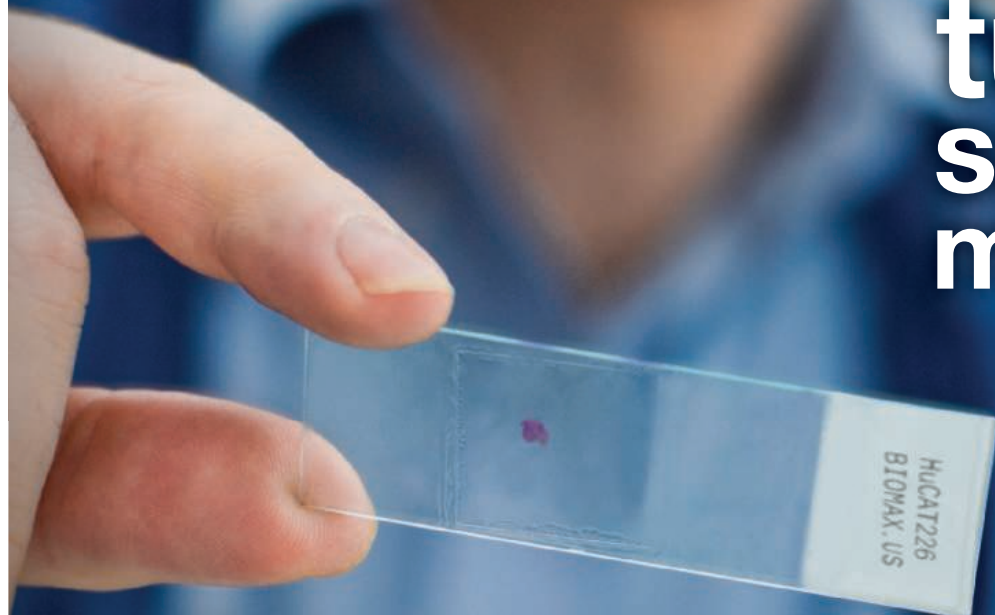
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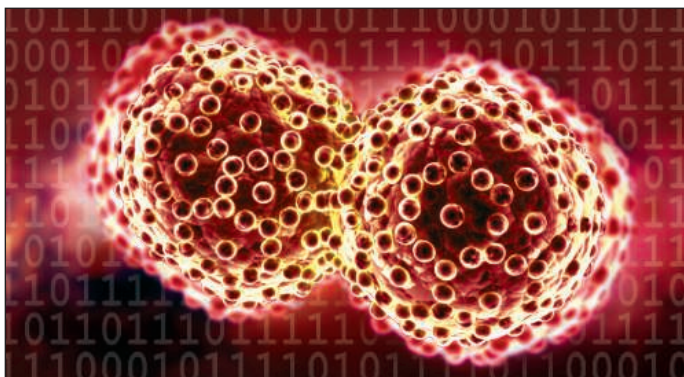
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A career in cancer research? Computational skills wanted

Cancer researchers are generating mounds of molecular data on tumor biology. Scientists with molecular and computational backgrounds are needed to move the growing field of precision oncology forward. Researchers with both skill sets, however, have a leg up. **By Gunjan Sinha**

When **William Pao** was a medical oncology fellow at Memorial Sloan Kettering Cancer Center (MSKCC) during the early 2000s, treating patients with metastatic non-small cell lung cancer (NSCLC) was rote: Every patient received the same chemotherapy regimen. But the odds of benefiting were hit or miss: “Only about 20% could expect to see their tumors shrink,” Pao says. “There was no way to know ahead of time who was going to benefit.” Even more nerve-racking for the patients was the fact that typically six weeks would pass before he could assess tumor response.

Today, depending on the mutations found in individual tumors, there are several unique drugs available to treat metastatic NSCLC. Therapies can be tailored, and for many patients the odds of surviving have gone from months to years. The success of “targeted therapies” in oncology—so named because they disable cancer cells in very specific ways—is being hailed a watershed moment in cancer therapy. Basic research on cancer mechanisms has led to over 40 targeted cancer therapies currently on the market, which take the form of monoclonal antibodies, small molecule drugs, and immunotherapies.

As targeted therapies take center stage in cancer treatment, they are profoundly changing the way research is done. Like many other medical research disciplines,

oncology is going molecular. The success of such drugs has fueled a push toward studying basic molecular mechanisms of cancer growth, which has brought with it “a crush of data so large that no human brain alone would be able to make heads or tails of it,” says **Levi Garraway**, assistant professor of medicine at the Dana-Farber Cancer Institute in Boston, Massachusetts. The complexity of the technology and tasks required to design targeted drugs and study their efficacy has grown so great that groups of scientists with varied expertise are required to continue to move the field forward, he adds. Although scientists working in the field today have largely picked up skills along the way, there will be a massive increase in demand for translational researchers with computational, analytical, and clinical trial expertise who can turn data into concrete knowledge. Future oncologists will need to have a much deeper understanding of tumor biology on a molecular level than their predecessors. “That is where the breakthroughs have been and will continue to be,” Garraway says.

The rise of molecular oncology

Lung cancer in particular has become the “poster child” for how research and treatment have changed, says **Charles Swanton**, a research oncologist at the Francis Crick Institute in London, England. The advent of therapies directed at tumors with mutations in epidermal growth factor receptor (*EGFR*), anaplastic lymphoma kinase (*ALK*), and B-Raf proto-oncogene (*BRAF*) genes over the past decade have dramatically changed outcomes, he says. These therapies were born out of a deep understanding of the potent genetic drivers of lung cancer, particularly in nonsmokers.

Pao was involved in studies of *EGFR* tyrosine-kinase inhibitors while at MSKCC, where he trained in medical oncology. He was part of a team that recognized that only about 10% of patients with NSCLC responded to the small molecule erlotinib—those whose tumors harbored mutations in the gene encoding *EGFR*. Those patients can benefit from the drug for years, says Pao. “Just being able to pinpoint patients has been a significant achievement.”

Pao’s career trajectory has paralleled the rise of molecular oncology. As a student at Yale University in New

Haven, Connecticut, he earned his M.D. and Ph.D. degrees and trained in a basic immunology lab. He then studied cancer cell signaling, discovering mechanisms of sensitivity and resistance to targeted agents. Today Pao heads the Oncology Discovery and Translational Area at Hoffman-La Roche, *cont.>*



William Pao

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Science For A Better Life

based in Basel, Switzerland. He is now tasked with developing new drugs that harness immune cells to attack cancer cells or target cancer cells directly. As part of its push toward precision oncology, the company announced early last year that it would spend \$1.03 billion to buy a 56.3% stake in Cambridge, Massachusetts-based Foundation Medicine, a company that uses genetics to help select drugs for cancer patients. One project involves probing Foundation Medicine's growing database of tumor profiles for specific mutations and using that information to either design drugs or to parse patients into clinical trials of the company's drugs, says Pao.

Similarly, **Wendy Winckler's** career path in oncology research "followed the genomic era," she says. Winckler is executive director of Next Generation Diagnostics at the Novartis Institutes for BioMedical Research in Cambridge, Massachusetts. She came to the company three years ago from the Broad Institute in Cambridge. Prior to that, she earned her Ph.D. in genetics at Harvard University and then landed her first job helping to build The Cancer Genome Atlas. She then became director of the Genetic Analysis Platform at Broad—a technology group that collaborated with both Broad and external scientists to generate and analyze diverse types of genomic data. "Having come from the science side, I was able to get experience in technology and also in being head of a large group."

Computational skills a plus

At Novartis, Winckler leads a department of 37 people. One early project was to help Genoptix—a Novartis daughter-company acquired in 2011 and located in Carlsbad, California—to commercialize a diagnostic test for lung cancer patients. That particular assay tests for "actionable mutations"—changes to those genes in a lung cancer sample that have been identified to date as helping guide treatment. Another major project is to characterize the tumors of patients across the company's ongoing cancer clinical trials to try to understand how genetic changes may influence response.

In Winckler's lab, about half of the scientists have computational expertise, and the other half have extensive wet lab skills. The most successful people, however, engage in both realms, she says. "Exposure to lab environments helps computational biologists have a more intuitive understanding of the data and an easier time planning sequencing experiments; lab scientists familiar with data analysis approaches can provide important insights while interpreting results."

Dual training has certainly benefited **Marcin Imielinski**, a molecular pathologist at Weill Cornell Medicine in New



"Having come from the science side, I was able to get experience in technology and also in being head of a large group."
—Wendy Winckler

York City. "Where I am right now is exactly where I hoped I would be," he says. "I feel like I have a single career instead of two, which I think is a big challenge for all M.D.-Ph.Ds."

Imielinski earned his undergraduate degree in computer science and then entered a newly christened M.D.-Ph.D. program in genomics and computational biology at the University of Pennsylvania in Philadelphia in 2001. He then chose pathology for his clinical training because he wanted "synergy between his research and his future clinical role," he says. At Weill Cornell, Imielinski is participating in the precision oncology effort, which will sequence tumor DNA to match patients to particular therapies.

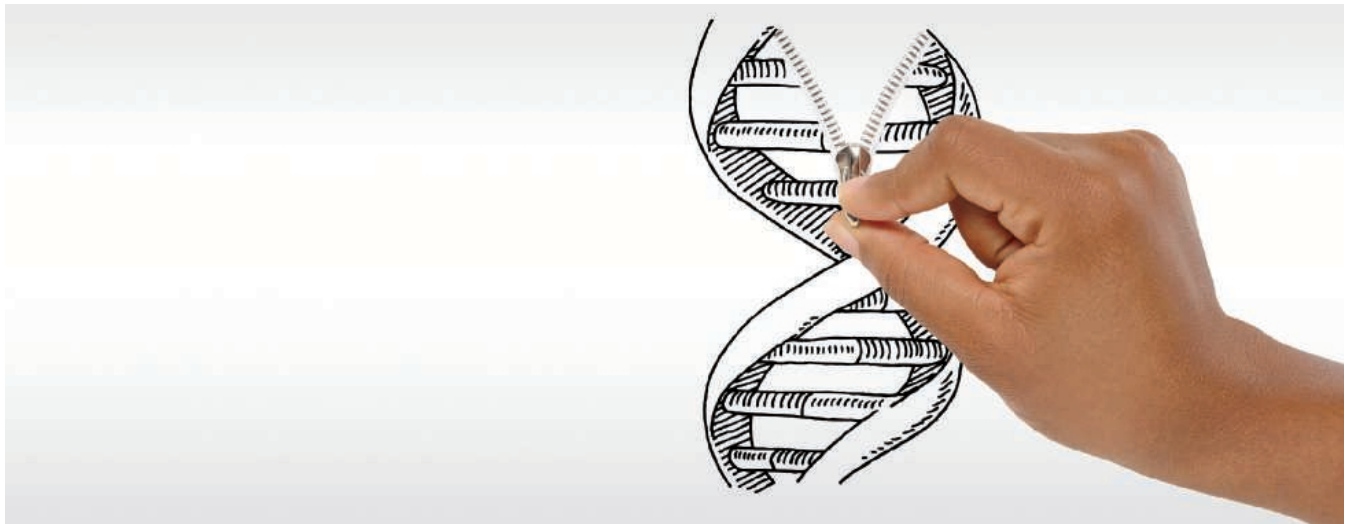
Imielinski is also building his own lab, which will focus on understanding the role of complex DNA rearrangements in cancer. Part of his task is to develop analytical and computational tools to make sense of the data. He also plans to leverage the latest sequencing technologies to understand how complex rearrange-

ments impact the tumor epigenome and perturb cancer genome structure over time.

Technology leads the way

Clearly, next-generation sequencing (NGS) technologies have had a trailblazing effect on career opportunities. Since the first targeted cancer therapy to treat chronic myelogenous leukemia became available in 2001, there has been an explosion in genomic technology. Where once it was possible to test tumor samples for only one mutation or genomic rearrangement at a time, NGS technology now enables testing for multiple gene mutations in multiple samples simultaneously. This technology has influenced not only the type and speed of cancer research being conducted, but it has also radically changed clinical practice. Recently, San Diego, California-based Illumina, one of largest manufacturers of sequencing machines, teamed up with Dana-Farber, MSKCC, and two other major U.S. cancer centers to define the "cancer actionable genome" to help tailor cancer therapies. Moreover, such technology is enabling research toward the next step in targeted therapy: understanding why cancers grow resistant to drugs.

At the Crick, Swanton's lab is focused on exactly that. In collaboration with the University College London (UCL) Cancer Trials Centre and the UCL Cancer Institute, Swanton's research group will be following about 850 patients with NSCLC from diagnosis to death as part of a clinical trial to understand tumor evolution. Tumors that are surgically removed as part of routine care will be dissected and different regions sequenced to build phylogenetic maps of the genomic events that drive cancer growth. **cont.>**



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About half of NSCLC patients go on to develop metastatic disease. As part of a second trial, Swanton's lab will also study what happens to tumors under the selective pressure of various types of cancer therapies, from traditional chemotherapy to targeted drugs.

As successful as targeted therapies are, "they aren't curing people," Swanton says. Resistance to therapy is inevitable. Research questions in his lab are driven by "what we see happening in human tumors," he says. The hope is that even better second- and third-generation therapies can be developed that can potentially limit the acquisition of resistance. "It's a very exciting time where laboratory-based molecular analyses are underpinning drug discovery and development."

At the German Cancer Research Center, (Deutsches Krebsforschungszentrum, or DKFZ) in Heidelberg, scientists working in precision oncology are also taking full advantage of advances in genomic technology. In 2012, the DKFZ formed the Heidelberg Center for Personalized Oncology (DKFZ-HIPO). The goal is to develop a clinical program for personalized oncology that will translate "the latest research and technologies from functional genomics and systems biology into clinical routine."

The initiative is "very ambitious," says **Roland Eils**, professor of functional genomics and bioinformatics and the codirector of DKFZ-HIPO. Of the several thousand cancer patients treated annually at the Heidelberg National Center for Tumor Diseases (NCT), whole-genome sequencing of tumors is offered to all patients who might benefit, says Eils. But DKFZ is taking research a giant step further. The cost of whole-genome sequencing is now comparable to exome sequencing, says Eils. So DKFZ-HIPO made a strategic decision to collect whole-tumor genome sequencing data for future research. If a patient consents, the sequence data is added to a database developed in-house. The database is designed to hold clinical annotations about patient progress and can perform multiple data analyses. So far, the database holds data from roughly 3,000 patients. The plan is to scale up to collecting whole-tumor genome sequence data from 3,000 to 4,000 patients annually. "That's an awful lot of data that poses a variety of challenges," says Eils. What's more, the researchers hope to be able to add other types of 'omics data such as RNA sequencing, epigenetic information, and histone modifications.

Obviously there is a huge need for experts in data management who have computational skills, Eils says. In collaboration with Heidelberg University, the DKFZ does indeed train many students and physicians in informatics and data management. But regrettably there is a demand for such experts in other industries such as banking that pay better, says Eils. Hopefully scientists will be drawn to work in cancer research because of "interest and excitement."

Precision medicine creates opportunities

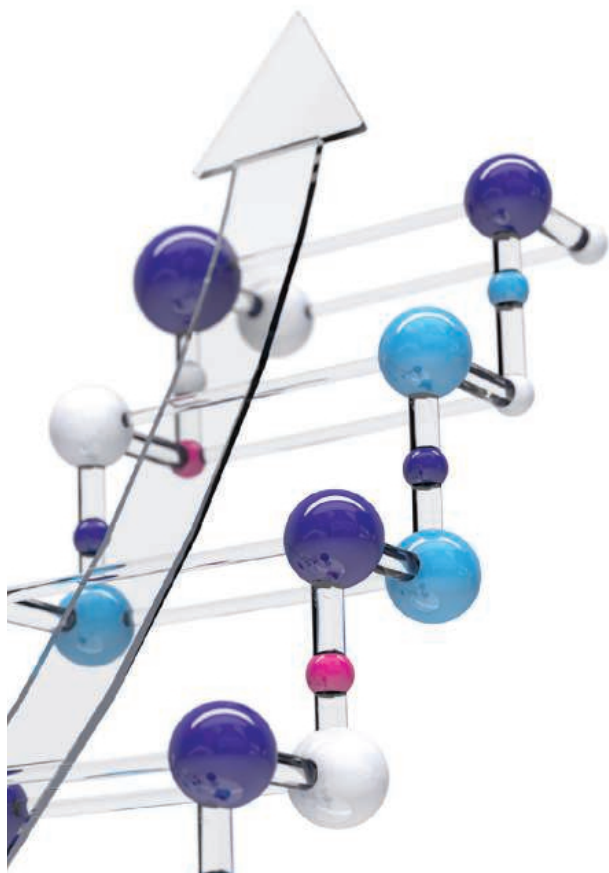
A candidate's enthusiasm about the future of cancer research is certainly a huge plus when Swanton is searching for new hires. Important questions he asks himself include, "How do they think? Does this person bring fascination and interest? How can they contribute?" With the goal to translate insights from his lab into the clinic, Swanton's lab holds a full spectrum of expertise, from "bench to bedside," including people with regulatory and clinical-trial experience.

The demand for such qualified people is set to grow, and not only within oncology. In January 2015, President Obama announced the Precision Medicine Initiative. Launched with a \$215 million investment in the President's 2016 budget, the initiative aims to "give clinicians tools to better understand the complex mechanisms underlying a patient's health, disease, or condition, and to better predict which treatments will be most effective," according to a statement issued by the White House. The bulk of the funding, \$130 million, will go to the National Institutes of Health to launch a population study that will follow 1 million Americans over many years to track their health; the National Cancer Institute will receive \$70 million to develop more effective personalized approaches to cancer treatment; the U.S. Food and Drug Administration will receive \$10 million to develop a database to advance innovation in precision medicine; and the Office of the National Coordinator for Health Information Technology will receive \$5 million to develop interoperability standards and address privacy and secure data exchange issues.

The amount of funding relative to the task is small, says **Mark Rubin**, head of the Institute for Precision Medicine at Weill Cornell Medicine and New York-Presbyterian Hospital, but "we are at the beginning," he adds. "That this is coming down from the government tells us that patients want access to their data to improve outcomes and that the government and regulatory agencies are going to make sure we find ways to make it happen." Moving forward, working in precision medicine could be an option for anyone with a scientific or mathematical background, he adds. For example, engineers who design devices will be needed as well as experts in population biology and epidemiology. "The opportunities will only expand."

Gunjan Sinha is a freelance writer living in Berlin, Germany.

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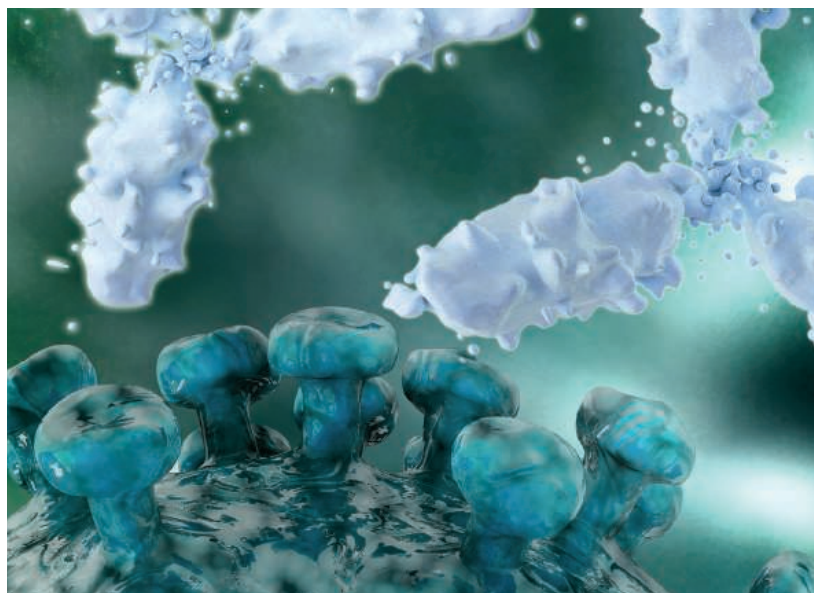
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All applications must conform to the Program Announcements and General Application Instructions that will be available for electronic downloading from the Grants.gov website (all viewable under CFDA number 12.420). Execution management support will be provided by the Congressionally Directed Medical Research Programs.

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Cleveland Clinic

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The Pediatric Institute of the Cleveland Clinic invites applications for new faculty positions at the Assistant or Associate Professor/Staff level. Exceptional candidates at the Professor/Staff level will also be considered. The positions will be based in the Lerner Research Institute of the Cleveland Clinic with a primary focus on basic or translational research. Applicants should have a M.D., Ph.D., D.V.M. or equivalent degree with postdoctoral training and show exceptional promise for an independent career in academic science. The Pediatric Institute is seeking individuals with a special interest in basic cellular and molecular processes, and/or translational studies.

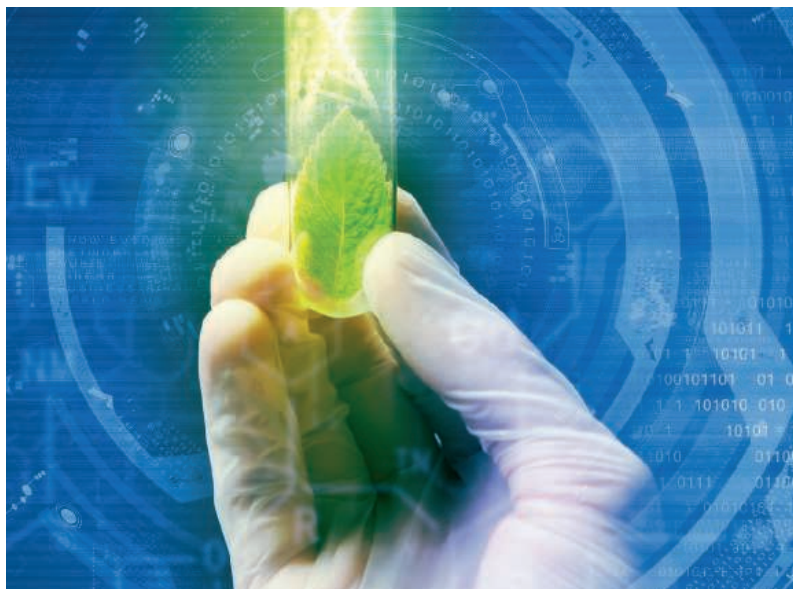
Areas of special interest include, but are not limited to:

- | | |
|------------------------|--------------------------|
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| ~ Kidney | ~ Autism |
| ~ Pediatric Care | ~ Microbiology |
| ~ Gastrointestinal | ~ Environmental Sciences |
| ~ Obesity and Diabetes | ~ Nanotechnology |
| ~ Microbiology | ~ Immunology |

The successful candidate is expected to establish an innovative, extramurally funded research program. The new faculty member will join a collaborative, interdisciplinary group of investigators. Interactions with excellent clinical programs are readily available and provide the potential to develop multi-disciplinary and translational research programs. The Lerner Research Institute has a strong tradition of collaborative research, and offers established programs for pre- and post-doctoral M.D. and Ph.D. trainees. Outstanding facilities and generous start-up funds are available. Salary will be commensurate with academic credentials and experience.

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The Department of Neurobiology and Anatomy at Wake Forest Medical School announces open positions for

Postdoctoral Training in Multisensory Processes

We seek strong candidates for postdoctoral training funded by an NIH T32 Training Grant. The training program provides a rich collaborative research environment that fosters interdisciplinary approaches to understanding how the brain integrates information from multiple senses to produce perception and adaptive behavior. Candidates with direct experience as well as those in related fields are encouraged to apply. Trainees will have access to any of 9 laboratories using human subjects and/or a variety of animal models (rodents-primates) with approaches spanning molecular/cellular to perceptual/behavioral. Fellowships are awarded on a competitive basis.

Applications including a current curriculum vitae or nominations should be sent to the Training Grant Director: **Dr. Barry E. Stein** (bestein@wakehealth.edu), or to its Co-Directors: **Dr. Terrence Stanford** (stanford@wakehealth.edu) and **Dr. Dwayne Godwin** (dgodwin@wakehealth.edu). A description of the faculty and the program can be accessed via the website: http://graduate.wfu.edu/admissions/t32/training_tpmp.html. Please also submit your application and CV to job opening **16654** at www.wakehealth.edu/jobsearch/.

EOE/AA: Minorities/Females/Disabled/Vets

UConn HEALTH

Postdoctoral Position Immunology

A postdoctoral position is available for a highly motivated individual interested in studying protective cellular immunity to respiratory virus infection. Candidates should have a Ph.D. in Immunology and solid experience in basic molecular biology, PCR analysis and flow cytometry. Specific areas of interest include tissue-specific lymphocyte migration, the role of antigen in protective immunity and signaling pathways that regulate homing-receptor expression. Candidates should have publications in peer reviewed journals in the field of immunology or infectious disease. Submit Curriculum Vitae with contact information for three references to:

Linda Cauley, Ph.D.
Department of Immunology, MC1319
UConn Health
263 Farmington Avenue
Farmington, CT 06030-1319
or via email: lcauley@uchc.edu
For further information refer to the UConn Health website at www.uchc.edu

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A researcher discovers teaching

When I had my first research experience, as a college student in a campus laboratory, I found that I loved collecting data to understand intriguing biological phenomena. I enjoyed exploring new ideas every day in the lab, and I appreciated the freedom I had to independently plan my experiments. So I decided that I wanted to be an academic scientist. My later scientific training left me well equipped to work in a lab—and that is where, 20 years later, I feel most comfortable. But it never prepared me for another responsibility that is now part of my job as a professor: teaching.

For the last 13 years, I have taught physiology to medical students, and until a few years ago, it left me very frustrated. I had a difficult time accepting that my students had little interest in scientific discoveries or landmark papers; they just wanted to cure people. Medical students expect to be taught by medical doctors, but I spent my days working in the lab instead of with patients, and I wondered how I could teach them anything valuable. I believed that my lack of enthusiasm for teaching was unfair to my students. But my frustration came mostly from seeing their grades. Their poor performance indicated that they were neither learning nor excited about the subject.

Amid this frustration, a new mentor came along who encouraged me to see teaching in a different light. She was working at my university on a sabbatical from her home institution, where she conducted research on education. I began working with her to explore this type of research for myself. At first I was hesitant, but when she told me that my classroom was my lab, I was intrigued. Although my graduate training hadn't taught me how to teach, it provided the foundation I needed to conduct research in this new discipline.

The research in turn motivated me to engage in teaching in ways that I never had before. I had doubted whether I had a teacher in me, but together, my mentor and I changed the dynamics of my classroom by introducing tools that emphasized active learning. Using electronic clickers and case studies encouraged student participation, and through class discussions, I could see that the students were striving to learn the material. They got excited, I got excited, and teaching started to be fun. I became a student again, this time trying to learn how people learn.



“Together, my mentor and I changed the dynamics of the classroom.”

contribution to science is modest. So today I ask myself: “Can I do more? Can I inspire my undergraduate students to learn? Can I help my own graduate students by showing them that teaching matters?” This is especially important to me as a professor in Mexico, because many Mexican Ph.D. holders cannot find research jobs and end up teaching full-time. They come into the world of teaching the same way I did: unprepared.

I don't plan to leave my scientific research, for now at least. Balancing my education work with my scientific research is challenging, but the satisfaction I gain from stimulating undergraduate students and encouraging graduate students to engage with teaching makes the struggle worthwhile. ■

Patricia Pérez-Cornejo is a professor at the Autonomous University of San Luis Potosí in Mexico. Send your story to SciCareerEditor@aaas.org.